Inflammatory cytokines compromise programmed cell death-1 (PD-1)-mediated T cell suppression in inflammatory arthritis through up-regulation of soluble PD-1.

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Inflammatory cytokines compromise programmed cell death-1 (PD-1)-mediated T cell suppression in inflammatory arthritis through up-regulation of soluble PD-1.

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PhD Immunology

Declaration

The work described here is my own, carried out personally unless otherwise stated. All sources of information, including quotations, are aknowledged by means of reference.

Davide Bommarito

Abstract

The programmed cell death-1 (PD-1) receptor is a key regulator of T cell activation and cytokine production. Multiple studies performed in PD-1-deficient mice demonstrate its importance in preventing autoimmunity. Evidence suggests that PD-1-mediated regulation is reduced during chronic inflammation in human diseases, such as rheumatoid arthritis (RA). In this thesis, the role of inflammation in influencing PD-1-mediated regulation of human CD4+ T cells is further characterised. First, PD-1 and PD-L1 expression, as well as the functional consequences of PD-1 ligation in rheumatoid (RA) and psoriatic arthritis (PsA) were analysed. Using flow cytometry and analysis of existing gene expression arrays, it was determined that the percentage of PD-1+ cells within the CD4+ and CD8+ T cells compartment was increased in RA and PsA synovial fluid (SF) compared to paired peripheral blood (PB). Upon *in vitro* T cell receptor (TCR) stimulation of HC CD4+ T cells in the presence of plate-bound PD-L1fc chimera, significantly decreased proliferation and interferon (IFN)-γ secretion was observed. In contrast, RA and PsA PB- and SF-derived CD4+ T cells appeared resistant to such PD-1-mediated inhibition. Second, it was investigated whether proinflammatory cytokines modulate PD-1-mediated regulation of healthy CD4+ T cells (HC). Addition of proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, which were increased in RA and PsA SF compared to HC and osteoarthritis (OA) controls, consistently abrogated PD-1-mediated suppression in HC CD4+ T cell cultures. Inhibitors of these cytokines reversed this effect. Finally, it was evaluated whether soluble PD-1 (sPD-1) negatively regulates the PD1/PD-L1 interaction. Soluble PD-1 (sPD-1) levels were increased in cell culture supernatants from TNFα and IL-6-stimulated cultures compared to untreated controls, and also in RA and PsA, but not in OA, serum and SF nor in HC

serum. qPCR analysis of HC CD4+ T cells from TNFα- and IL-6-stimulated cultures also revealed increases of the PD-1Δex3 splice variant. Functionally, addition of sPD-1fc counteracted PD-1-mediated suppression of HC CD4+ T cells, increased T cell proliferation in HC CD4+ T cell/monocyte co-cultures but had no effect on HC CD4+ Treg cell-mediated suppression. Together, the data presented in this thesis provide new evidence that the inflammatory environment of the RA and PsA joint compromises PD-1/PD-L1 mediated T cell regulation.

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List of Abbreviations

ACPA	Anti-citrullinated protein antibody
ADA	Adalimumab
AE	Adverse event
APC	Antigen presenting cells
APC	Allophycocyanin
AS	Ankylosing spondylitis
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collage-induced arthritis
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
CCP	Cyclic citrullinated peptide
CRE	Cis regulatory element
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CTV	Cell trace violet
DC	Dendritic cell
DMSO	Dimetylsulfoxide
DAS	Disease activity score
DMARD	Disease modyfing antirheumatic drug
EAE	Experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor

ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FAO	Fatty acid β-oxidation
FMO	Fluorescence minus one
FoxO1	Forkhead box protein O1
FoxP3	Forkhead box P3
GATA	GATA binding protein
GMCSF	Granulocyte/macrophage colony-stimulating factor
HC	Healthy control
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
IA	Inflammatory arthritis
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IRF	IFN-regulatory factor
IL-17	Interleukin-17
IL-6	Interleukin-6
IL-1β	Interleukin-1-beta
ISG	Interferon-stimulated gene
ISRE	IFN-stimulated response element
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
iTregs	Induced Tregs
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis

KO	Knockout
LAG-3	Lymphocyte-activation gene 3
LAIR	Leukocyte-associated Ig-like receptor
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mechanistic target of rapamycin
MTX	Methotrexate
NFATc1	Nuclear factor of activated T-cells cytoplasmic 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD	Non-obese diabetic
OA	Osteoarthritis
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PE	Phycoerythrin
PI3K	Phosphatidylinositol-3-kinase
PK	Protein kinase
PRDM1	PR domain zinc finger protein 1

PsA	Psoriatic arthritis
PTEN	Phosphatase and tensin homolog
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROR	RAR-related orphan receptor
SEM	Standard error of the mean
SLE	Systemic lupus erithematosus
SNP	Single nucleotide polymorphism
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SJC	Swollen joint count
STAT	Signal transducer and activator of transcription
sPD-1	Soluble PD-1
SyK	Spleen tyrosine kinase
TCR	T cell receptor
Teff	T effector cells
TGF-β	Transforming growth factor beta
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumour-infiltrating lymphocyte
TIM-3	T cell/transmembrane, immunoglobulin, and mucin 3
TJC	Tender joint count
TNFα	Tumour necrosis factor alpha
TOC	Tocilizumab

Treg	T regulatory cell
UTR	Untranslated region
ZAP	Zeta-chain associated protein

1 Introduction

This introduction will begin with a brief description of effector T cell subsets and the process of T cell activation and regulation. Next, I will introduce a number of different T cell inhibitory receptors including CTLA-4, PD-1, TIGIT, TIM-3 and LAG-3. Since this thesis focuses on the role of PD-1-mediated regulation in human rheumatoid and psoriatic arthritis, a comprehensive review of the literature regarding the PD-1/PD-L1 pathway will be presented. This will include detailed descriptions of: i) the gene and protein structure of PD-1 and its ligands, ii) PD-1 transcriptional regulation, iii) PD-1 and PD-1 ligands expression, iv) the effects of PD-1 on T cell signalling and T cell metabolism, v) the effect of reverse signalling through the PD-1 ligand PD-L1. Since PD-1 function has been extensively investigated in human cancer but only partially in human autoimmunity, the role of PD-1 in cancer immunotherapy will be briefly discussed. This will be followed by a more detailed analysis of current literature outlining the development of autoimmune-like adverse effects following PD-1 blockade as well as the role of PD-1 in autoimmunity and in mouse and human arthritis. Finally, a brief description of the clinical aspects of rheumatoid and psoriatic arthritis will be provided, followed by the rationale and aims of this thesis.

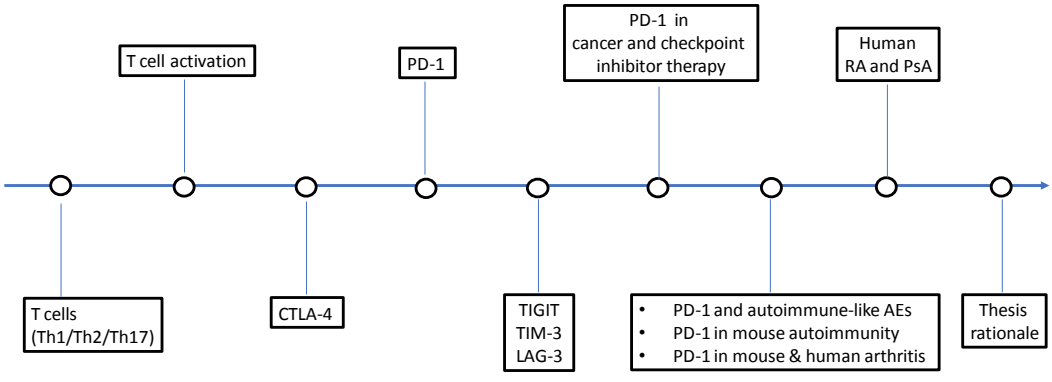

Figure 1.1 Schematic layout of the literature described in the introduction

1.1 T cells

A key feature of the immune system is the ability to distinguish self from non-self. This process begins with recognition and binding of a T cell receptor (TCR) to an antigen displayed by the major histocompatibility complex (MHC) expressed on the surface of an antigen-presenting cell (APC). Multiple factors then come in place to influence whether this process leads to T cell activation or anergy. In the thymus, T cells proliferate and develop a repertoire of TCRs by recombining TCR gene segments. T cells displaying reactivity to self-peptides undergo deletion in the thymus (1). This process called central tolerance helps to prevent the development of autoimmunity (2). T cells lacking the ability to bind to MHC molecules undergo apoptosis. T cells that can respond to MHC molecules mature in the thymus before entering the periphery. Importantly, some TCRs may have a specificity that is cross-reactive with self-antigens. From the thymus, naïve T cells circulate through the blood, spleen and lymphatic organs. In the periphery T cells are exposed to APCs displaying foreign antigens, in case of infection, and both self or mutated self-molecules in the case of malignancy (2). CD8 and CD4 are two transmembrane glycoproteins that serve as co-receptors and which bind to the invariant parts of MHC class I and II, respectively, stabilising the MHC-peptide-TCR complex during T cell activation. CD8+ T cells have cytotoxic functions, enabling killing of target cells such as infected cells or cells that have undergone malignant transformation (3). CD4+ T cells, also known as T "helper" cells, are fundamental for adaptive immune responses. They can i) activate B cells to secrete antibodies, ii) help macrophages to clear pathogens and iii) support cytotoxic T cells to clear infected and transformed cells. At present, the best-characterised T helper (Th) cell subsets are Th1, Th2 and Th17 cells

but Regulatory T cells (Treg) and follicular T helper cells (Tfh) are becoming highly important in many biological scenarios (4).

1.2 Th1, Th2 and Th17 cells

Th1 cells are activated in response to infection mediated by intracellular pathogens and produce specific cytokines including interferon-gamma (IFNγ), interleukin-2 (IL-2), lymphotoxin, and tumour necrosis factor alpha (TNFα). These cytokines are important for T cell proliferation and to enhance the phagocytic and antigen presenting capacity of macrophages. Th1 cells are also able to promote complement-fixing and opsonising antibodies, specifically immune-globulin G1 (IgG1) in humans and IgG2a in mice (4). Th2 cells are induced in the presence of extracellular pathogens (i.e. nematodes and helminths). During Th2 responses, these cytokines induce B cell activation and class switching to IgE and subtypes of IgG. Th2 cell-derived cytokines can also stimulate mast cells and eosinophil promoting their activation and leading to antibody and allergic responses (4). Th17 cells play an important role in adaptive immunity during infection by extracellular bacteria and fungi. Like other T helper cells, Th17 cells can interact with B cells in response to pathogens and they are involved in B cell recruitment via the CXCL13 chemokine signalling (4-6). Genetic defects associated with development of Th1, Th2 and Th17 cell subsets lead to different pathologies and clinical manifestations (7, 8).

For example, abnormal activation of Th1 and Th17 cells is typically found in a variety of autoimmune diseases. Th1 and Th17 dysregulation is associated with disease pathogenesis via production of proinflammatory cytokines such as interleukin-17A (IL-17A), interferon-gamma (IFNγ) and tumour necrosis factor-alpha (TNFα) (9-12). Work from our lab has demonstrated that IL-17+, IFNγ+ and TNFα+ CD4+ T

cells are abundantly represented at sites of inflammation (synovial fluid and tissue) in patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA) (13-15).

1.3 Differentiation of naïve CD4+ T cells into Th1, Th2 and Th17 subsets

TCR-stimulated naïve CD4+ T cells differentiate into different subpopulations following specific cytokines stimuli and following activation of transcription factors of the Signal Transduction and Activator of Transcription (STAT) family. Naïve CD4+ T cells are driven towards a Th1 phenotype by the cytokines IFNγ and IL-12 which signal through STAT1 and STAT4, respectively (16). This is followed by expression of the T-bet transcription factor, which induces production of IFNγ, the hallmark cytokine of the Th1 subset (17). Th1 also produce IL-2 and TNFα (18).

Th2 differentiation is driven by IL-4 signalling via STAT6 together with TCR stimulation. This combined stimulation leads to expression of GATA binding protein 3 (GATA-3) and production of cytokines IL-4, IL-5, IL-9 and IL-13 (18-20). IFNγ and IL-4 are known to amplify Th1 and Th2 differentiation, respectively, through an autocrine mechanism but they are also capable of antagonising one another. For example, the Th1 hallmark cytokine IFNγ can activate STAT1 leading to expression of T-bet (19). T-bet is a repressor of Th2 cell differentiation and when transduced in polarised Th2 cells can modulate their phenotype towards the Th1 profile (15).

CD4+ T cell differentiation into Th17 cells requires stimulation via the cytokines transforming growth factor-beta (TGF-β) and IL-6. IL-6 signals through STAT3 which promotes the expression of the gene encoding retinoic acid receptor-related orphan receptor-γt (RORγt; the master transcriptional regulator; encoded by *Rorc*). This leads to expression of the Th17 lineage specific cytokine IL-17 (21, 22). Other cytokines including IL-1β, TNFα and IL-23 can promote or amplify Th17

differentiation (22). Th17 cells can produce proinflammatory cytokines TNFα, IL-17F, IL-21 and IL-22 (10). In addition to their signature cytokines and specific transcription factors, Th1, Th2 and Th17 subsets express lineage specific chemokine receptors. For example, within CXCR3+CD4+ the most represented subset is Th1 while CCR4+CD4+ T cells contain Th2 cells. Conversely, the CCR6+ CCR4+ subset contains predominantly Th17 and a small percentage of Th1 cells (23).

1.4 T cell activation and T cell inhibitory pathways

During the first step of T cell activation the TCR binds to the MHC/peptide complex. A second step in T cell activation is the binding of CD80 (B7-1) and CD86 (B7-2) molecules expressed on the APC to CD28 molecules expressed on the T cell. This second step provides a co-stimulatory signal important for cell activation. Following TCR:MHC and CD28:CD80/86 interactions, several intracellular pathways become activated leading to cell proliferation, increased expression of cell survival genes and cell differentiation through the production of growth cytokines such as IL-2 and IFNγ (24). Several receptors, functioning as immune checkpoints are in place to downregulate immune responses, prevent uncontrolled immune activation, reduce inflammation and maintain peripheral self-tolerance (2, 25).

The two best-characterised inhibitory receptors are the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, CD152) and the programmed cell death 1 (PD-1, CD279) (26) receptor but multiple additional molecules have been discovered in recent years and represent promising targets for therapeutic manipulation. These molecules include the T-cell immunoglobulin and ITIM domain (TIGIT), the lymphocyte activation gene 3 (LAG-3) and the T-cell Ig and mucin domain-3 (TIM-3). In the next sections the inhibitory receptors CTLA-4, PD-1, TIGIT, TIM-3 and

LAG-3 will be described. Stronger attention will be dedicated to PD-1 receptor and its involvement in cancer and autoimmunity.

1.5 Immune checkpoints: CTLA-4

CTLA-4 is an essential negative regulator of T cell immune responses. CTLA-4 is expressed by T cells and regulates the magnitude of the early stages of T-cell activation in the lymphoid organs (27-29). Specifically, CTLA-4 stops autoreactive T cells within the lymph nodes at the initial stage of naïve T-cell activation by both outcompeting CD28 in binding CD80 and CD86, as well as actively delivering inhibitory signals to the T cell (25, 30). CTLA-4 is a homolog of CD28 but has a higher binding affinity for CD80 and CD86. Upon CTLA-4 engagement with CD80 and CD86, several mechanisms play a role in reducing T cell activation. CTLA-4 is able to inhibit directly the TCR immune synapse, it can modulate the CD28-mediated signalling pathway and it has been shown to increase T cell mobility making it harder for T cells to interact with APCs (31, 32). Qureshi *et al.* elegantly showed that CTLA-4 is also able to capture its CD80 and CD86 ligands from opposing cells by a process called trans-endocytosis. Removed costimulatory ligands are then degraded inside CTLA-4-expressing cells resulting in reduced costimulation via CD28 (33).

CTLA-4 delivers an inhibitory signal within the T cell blocking IL-2 production and inhibiting the cell cycle (25, 34-36). Evidence also suggests that whether a cell will undergo activation or anergy is determined by the relative amount of CD28:B7 binding versus CTLA-4:B7 binding (30). CTLA-4 expression on the surface of T cells can be regulated by the intensity of the TCR and CD28:CD80/CD86 signalling. In resting naïve T cells CTLA-4 is located primarily in intracellular vesicles and cell surface expression of CTLA-4 rapidly increases by rising of

intracellular calcium levels. Furthermore, during T cell activation, intracellular and cell surface CTLA-4 re-direct towards sites of TCR activation (37).

Regulatory T cells (Treg) are important players during immune responses and their role in maintaining peripheral tolerance has been extensively demonstrated (38, 39). Treg cells constitutively express CTLA-4 and use this molecule during suppressive functions (39, 40). Several authors have shown that CTLA-4 deficiency in Tregs leads to impaired suppressive capacity and that Tregs are able to control effector T cells activation by binding and downregulating CD80 and CD86 ligands on APCs, leading to reduced CD28 costimulation (33, 39, 40). Interestingly, T cell regulatory ability via CTLA-4-mediated regulation is not a unique feature of Treg cells and CTLA-4 transfection in resting human CD4+CD25- T cells has been shown to confer suppressive capacity in absence of the forkhead box P3 (FoxP3) protein (41).

The critical role for CTLA-4 in immune tolerance is demonstrated by the fact that CTLA-4 knockout mice rapidly develop a fatal T cell–mediated multiorgan inflammation similar to a systemic autoimmune disease (42, 43). CTLA-4 is essential in prevention of autoimmunity and blockade of this molecule has been shown to exacerbate autoimmunity in different mouse models including Systemic Lupus Erithematosous (SLE), multiple sclerosis (MS), and type 1 diabetes (44). In human, polymorphisms in the *CTLA-4* gene are associated with autoimmune diseases including type 1 diabetes, Graves' disease and RA while heterozygous CTLA-4 mutations result in reduced CTLA-4 mRNA and CTLA-4 protein expression and to severe immune dysregulation, defective Treg function, hyperproliferative T cells and lymphocytic infiltrates in nonlymphoid organs (45-47).

The critical role of CTLA-4 in controlling T cell activation has led to the development of a recombinant fusion protein (abatacept) comprising the extracellular

domain of human CTLA4 and a fragment of the Fc domain of human IgG1. This molecule is able to compete with CD28 for CD80 and CD86 binding to selectively modulate T-cell activation and is currently used for the treatment of RA (48, 49) and juvenile idiopathic arthritis (JIA) (50).

CTLA-4 is also an important target for anti-tumour therapies. In murine tumour models, anti-CTLA-4 antibodies have been shown to be highly efficient in promoting antitumor immune responses (51-53). The therapeutic effect of anti-CTLA-4 antibodies is likely due to two mechanisms. The first mechanism is blocking the interaction between CTLA-4 on T cells and B7 ligands (CD80/CD86) on APCs. The second mechanism may operate by blocking CTLA-4 on Treg cells and depleting intra-tumoural CTLA-4+ Treg cells via antibody-dependent cellular cytotoxicity (52, 53). These studies led to clinical development of anti-CTLA-4 blocking antibodies for cancer therapy and anti-CTLA-4 treatment (ipilimumab) is now approved as a treatment for metastatic melanoma (54, 55).

1.6 Immune checkpoints: PD-1

1.6.1 Gene and protein structure of PD-1

The Programmed Cell Death 1 (PD-1) protein was first described in 1992 as an upregulated gene in a T cell hybridoma undergoing cell death (56). In 1999, Nishimura *et al.* demonstrated that PD-1 has important negative regulatory functions and showed that lack of the receptor in the *Pdcd1*$^{-/-}$ mice phenotype led to the development of autoimmunity including lupus-like autoimmune disease and autoimmune dilated cardiomyopathy (57, 58). The identification of the PD-1 ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (59-62) has been paramount in understanding the functions of the PD-1 signalling pathway. In mouse, the *Pdcd1* gene

is located on chromosome 1 while in human the PD-1 gene (*PDCD1*) is located on chromosome 2. In both species, the PD-1 gene is composed of 5 exons. The exon 1 and 2 encode a short signal sequence, and the IgV-like domain, respectively. Exon 3 encodes the stalk and transmembrane domains while exon 4 encodes a short sequence of 12 amino acids (aa) marking the beginning of the cytoplasmic domain. The C-terminal intracellular residues and the 3'UTR are encoded by exon 5. Five splice variants of the PD-1 gene have been identified in activated human T cells. These transcripts lack exon 2 only, exon 3 only, exons 2 and 3, or exons 2 through 4 and they can be induced following anti-CD3/CD28 stimulation (63). Interestingly, the transcript lacking exon 3 (PD-1Δex3) encodes a protein lacking the transmembrane domain and strongly resembling the soluble form of CTLA-4, a molecules that plays an important role in autoimmunity (47).

PD-1 is a member of the CD28/CTLA-4 superfamily having 15% aa identity with CD28, 20% identity with CTLA-4, and 13% identity with inducible T cell costimulator (ICOS). The receptors in the CD28/CTLA-4 superfamily are all type I transmembrane glycoproteins composed of three major parts: i) an Ig Variable-type (V-type) extracellular domain, ii) a transmembrane domain, and iii) a cytoplasmic tail responsible for the binding of signalling molecules (Figure 1.3). PD-1 is a 288 aa protein composed of an extracellular signal sequence, an immunoglobulin IgV-like domain, a 20 aa stalk, a transmembrane domain and a cytoplasmic domain (see Figures 1.2 and Figure 1.3B). PD-1 cytoplasmic tail contains the N-terminal sequence VDYGEL which forms an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM). PD-1 cytoplasmic tail also contains the C-terminal sequence TEYATI, which forms an Immunoreceptor Tyrosine-based Switch Motif (ITSM) (Figure 1.2) (64). CD28,

CTLA-4, and ICOS are covalently linked homodimers due to the interchain disulfide mediated by the equivalent of Cys122 in CTLA-4 (65). In contrast, PD-1 cannot form such a covalent dimer as it lacks the analogous Cys residue. In addition, PD-1 possesses only a single intrachain disulfide in its Ig V-type domain, while CD28, CTLA-4, and ICOS contain two intrachain disulfides. Another specific feature of PD-1 is that the complementarity-determining region 3 (CDR3) loop of the receptor does not contain the conserved XXPPP (F/Y) motif present in CD28, CTLA-4, and ICOS. Fluorescence resonance energy transfer (FRET) analyses of full-length PD-1 and analytical ultracentrifugation on a soluble extracellular PD-1 IgV-like domain showed that PD-1 is monomeric (66). PD-1 has other distinct molecular features compared to the members of the CD28 family (67). Specifically, CD28, CTLA-4, and ICOS have SH2-binding motifs (YxxM) located in their cytoplasmic tails. CTLA-4 and CD28 also have one and two SH3-binding motifs (PxxP), respectively, in their cytoplasmic tails. On the contrary, the PD-1 receptor has no SH2- or SH3-binding motifs in its cytoplasmic tail but is still able to recruit SH2 domain–containing phosphatases (67).

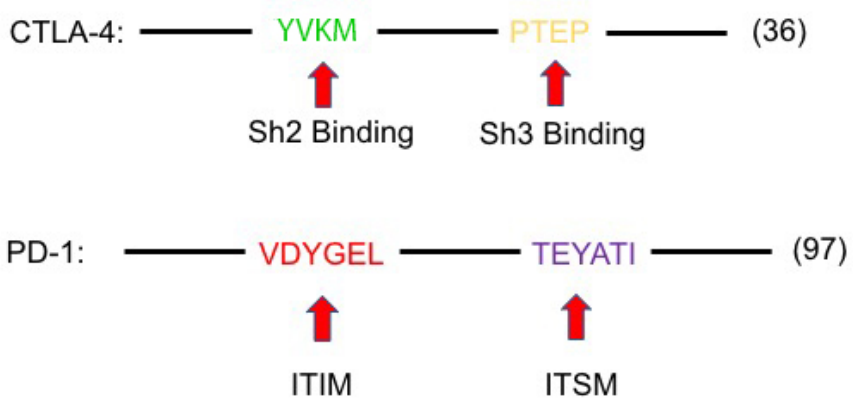

Figure 1.2 Schematic diagram showing the cytoplasmic tail of CTLA-4 and PD-1.
YVKM (Sh2 binding domain), PTEP (Sh3 Binding domain), VDYGEL (ITIM) and TEYATI (ITSM). Adapted from (68).

The cytoplasmic tail of the PD-1 receptor has important signalling functions. Chemnitz *et al.* demonstrated that PD-1-mediated inhibitory effect on the PI3K signalling pathway is lost when the ITSM tyrosine is changed to phenylalanine (Y248F) but remains functional when this change affects the ITIM tyrosine (Y223F) (69). Using mass spectrometry it was found that the ITSM motif of PD-1 acts as a docking site of Src homology 2 domain–containing tyrosine phosphatase 2 (SHP-2) and possibly Src homology 2 domain–containing tyrosine phosphatase 1 (SHP-1) although the role of SHP-1 remains unclear (69, 70). This was further demonstrated using live-cell imaging during T cell activation by Yokosuka *et al.*, which demonstrated that only SHP-2 interacts with PD-1 in the selected experimental model (71). During T cell activation, the PD-1 receptor clusters with T cell receptors (TCRs) upon binding with its ligand PD-L1 and is transiently associated with the phosphatase SHP-2. These negative costimulatory micro-clusters dephosphorylate the proximal TCR signalling molecules modulating cell proliferation and cytokine production (71).

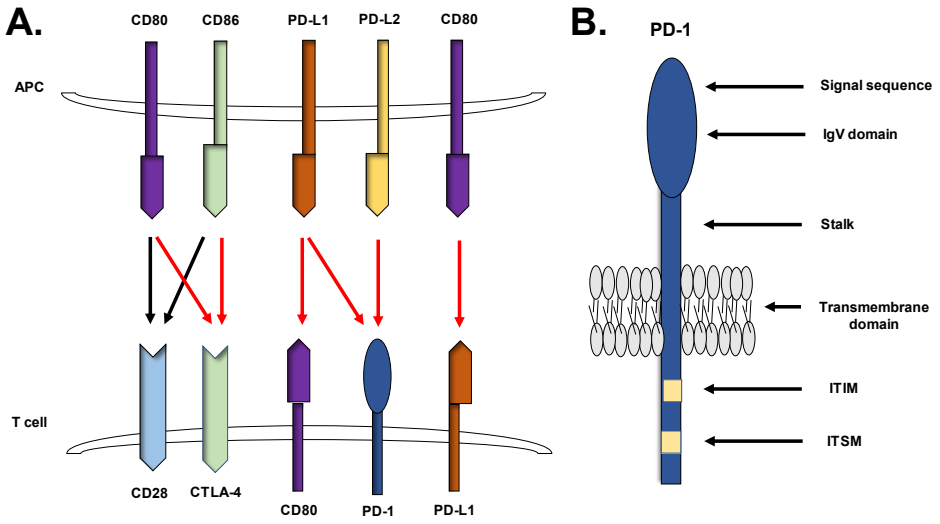

Figure 1.3 B7–CD28–CTLA-4 costimulatory molecules and the structure of programmed cell death 1 (PD-1).
(A) Members of the B7-CD28-CTLA-4 family of costimulatory molecules. Black arrows indicate stimulatory signals and red arrows indicate inhibitory signals. (B) Structure of human PD-1 showing the signal sequence, the immunoglobulin variable region (IgV)-domain, the transmembrane domain and the ITIM and ITSM domains.

1.6.2 Gene and protein structure of PD-1 ligands PD-L1 and PD-L2

PD-L1 is the best-characterised ligand for PD-1. PD-L1 is a 290 aa type I transmembrane protein which in mice, is encoded by the *Cd274* gene located on chromosome 19 and in human is encoded by the *CD274* gene located on chromosome 9. *CD274* is composed of seven exons. Exon 1 is non-coding and contains the 5'UTR. Exons 2, 3 and 4 contain the signal sequence, IgV-like domain, and IgC-like domains, respectively. The transmembrane domain and the intracellular domains are encoded by exons 5 and 6, respectively. Exon 7 encodes intracellular domain residues and the 3'UTR. The intracellular domain of PD-L1 is composed by 30 aa and it is highly conserved among different species. In humans, only one splice variant has been reported for PD-L1. This splice variant lacks the IgV-like domain encoded in exon 2 and theoretically should not be able to bind PD-1 although its function has not been investigated (72). No splice variants have been identified for PD-L1 in mouse.

PD-L2 is the second ligand of PD-1 (61). PD-L2 is a type I transmembrane protein encoded by the *Pdcd1lg2* gene which is adjacent to the *Cd274* gene in both mouse and human. The two genes are separated by only 23 kilobase (kb) of intervening genomic DNA in mouse and 42 kb in human. In mouse, the *Pdcd1lg2* gene located on chromosome 19. In human, the *PDCD1LG2* gene is located on chromosome 9.

The PD-L2 gene is composed of six exons in mouse and seven in human. Exon 1 and 6 are non-coding, while exon 2 encodes a signal sequence. Exon 3 and 4 encode for the IgV-like domain, the IgC-like domain respectively, while exon 5 encodes for a short stalk, the transmembrane region and the beginning of the cytoplasmic domain. A stop codon in the mouse exon 5 results in a cytoplasmic domain of 4 aa. In human, exon 6 and 7 encode for an additional coding region resulting in a longer cytoplasmic

domain of 30 aa as compared to the mouse domain. The long form of the cytoplasmic domain of PD-L2 has no signalling motifs but is conserved across diverse species, including human, macaque, chimp, dog, cow and pig suggesting a possible functional role for the cytoplasmic tail of PD-L2. Three different splice variants for PD-L2 have been identified following activation of human PBMC (61, 73). The first variant lacks the exon 3 and since PD-L2 binding activity resides in the IgV-like domain (74) this truncated form is unlikely to bind PD-1 (61). A second form lacks the IgC-like domain and it is thought to be still able to bind PD-1 although this has not been further investigated. Finally, a third variant lacks both the IgC-like and the transmembrane domains but maintains the intracellular residues leading to a soluble form of the PD-L2 ligand.

1.6.3 Transcriptional regulation of PD-1 expression

Cis-regulatory elements (CREs) such as enhancers and promoters are regions of non-coding DNA that regulate the transcription of nearby genes. Genetic mutations affecting the function of these sequences are responsible for phenotypic diversity (75). Several *cis*-elements have been identified as regulators of PD-1 gene expression. By using two models consisting of murine EL4 cells, which constitutively express PD-1, and primary murine CD8+ T cells that express PD-1 upon T cell stimulation, Oestreich *et al.* identified two tissue-specific hypersensitive sites at the 5′ conserved region of the PD-1 locus. The two conserved regions (*CR-B* and *CR-C*) are associated with *Pdcd1* activation, they contain multiple transcription factor binding sites and are located 100 bp and 1.1 kb upstream of the transcription start site (TSS) (76). A binding site for the activator protein 1 (AP-1) is encoded within *CR-B* (77) while *CR-C* contains an IFN-stimulated response element (ISRE) (78), a nuclear factor of

activated T-cells cytoplasmic 1 (NFATc1) binding site (76), a Forkhead box protein O1 (FoxO1) binding site (79), and a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding site (80). Interestingly, it was shown that PD-1 expression is not induced by constructs containing the PD-1 promoter but lacking the *CR-C* region. This suggests that the *CR-C* region is critical for PD-1 expression (76, 80). In mouse-derived T cells, several transcription factors have been shown to modulate PD-1 expression.

One example is NFATc1. This transcription factor is necessary for initial activation-induced expression of PD-1 in CD4+ and CD8+ T cells. TCR activation leads to activation of the calcineurin pathway and NFAT1c (81). Following activation, NFAT1c translocate to the nucleus and binds strongly to the *CR-C* region of the PD-1 gene regulating PD-1 expression in T cells. The role of NFATc1 as inducer of PD-1 is further demonstrated by the fact that the NFATc1-specific inhibitor peptide VIVIT and cyclosporine A, an inhibitor of calcineurin, are able to abrogate PD-1 expression (76). AP-1 is another transcription factor that positively regulates PD-1 expression. TCR-mediated stimulation leads to the activation of the MAPK cascade and AP-1 (82). Using a lung carcinoma tumour model, Xiao *et al.* demonstrated that overexpression of the AP-1 subunit c-Fos induces PD-1 expression in CD4+ and CD8+ T cells resulting in the inhibition of antitumor T cell-mediated responses. Conversely, a mutation at the AP-1 binding site reduced PD-1 expression in tumour-infiltrating T cells leading to increased tumour clearance (77). The Notch signalling pathway is also capable of inducing PD-1 expression. Using specific inhibitors of this pathway, Mathieu *et al.* showed that *in vitro* blockade of the Notch pathway had a moderate, dose-dependent reduction of PD-1 expression and *Pdcd1* transcription in

activated CD8+ T cells, without affecting the overall activation of the T cells (83). FoxO1 is a transcription factor known to promote transcription of genes necessary for homeostatic maintenance of naïve and memory T cells (84). Starron *et al.* demonstrated that FoxO1 binds to the *CR-C* region of the *Pdcd1* locus and that it is necessary to generate PD-1^{hi} T cells in a chronic murine lymphocytic choriomeningitis virus infection model. On the contrary, it was shown that FoxO1 knockout mice had lower PD-1 expression compared to controls (79).

The two best-characterised inhibitors of PD-1 are T-bet, the transcription factor encoded by T-box transcription factor *TBX21*, and the PR domain zinc finger protein 1 (*PRDM1*) also known as Blimp-1. T-bet was the first identified inhibitor of PD-1 (85). T-bet bounds directly to a regulatory region upstream of the *Pdcd1* transcriptional start site. Kao *et al.* examined the role of T-bet in regulating virus-specific CD8+ T cell responses during chronic viral infection in mouse and showed that T-bet expression is downregulated in exhausted CD8+ T cells in response to persisting antigen. Furthermore, T-bet knockout mouse was shown to have exacerbated CD8+ T cell exhaustion and increased viral load while overexpression of T-bet strongly reduced the expression of PD-1 (86). Similarly to T-bet, Blimp-1 is also capable of negatively regulating PD-1 expression in mouse. Lu *et al.* showed that Blimp-1-deficient CD8+ T cells fail to repress PD-1 during the early stages of CD8+ T cell differentiation after acute infection with lymphocytic choriomeningitis virus. Blimp-1 represses PD-1 via two different mechanisms. Firstly, it negatively regulates PD-1 directly and secondly, it is able to displace the PD-1 activator NFATc1 from its binding site (87).

Further indication that Blimp-1 is a negative regulator of PD-1 comes from a study showing that T follicular helper cells (T_{FH}) express high levels of PD-1 but they lack Blimp-1. In this study, exogenous expression of Blimp-1 abrogated the PD-1^{hi} T_{FH} cell subsets indicating an inverse correlation between Blimp-1 levels and PD-1 expression (88). In contrast with the reported data, Blimp-1 might also act as an inducer of PD-1 under specific circumstances. In a mouse model of lymphocytic choriomeningitis virus (LCMV) chronic viral infection Blimp-1 was expressed in exhausted PD-1+CD8+ T cells while in exhausted CD8+ T cells from a Blimp-1 knockout mice, PD-1 expression was slightly reduced compared to the control suggesting a different role for Blimp-1 in regulating *Pdcd1* in these experimental conditions (89).

Studies focusing on murine macrophages have also identified NF-κB and interferon-stimulated gene factor 3 (ISGF3) as two inducers of PD-1 in this specific cell population. Bally *et al.* found that the NF-κB p65 subunit binds to a site in *CR-C* region and that NF-κB was able to induce PD-1 expression following TLR stimulation. Importantly, blocking NF-κB binding to the DNA using an inhibitor was shown to abrogate PD-1 expression (80). PD-1 regulation in macrophages following cytokine stimulation was also examined. Cho *et al.* showed that following interferon alpha (IFNα) stimulation of macrophages, the IFN-stimulated gene factor (ISGF3) complex, which is composed by STAT1, STAT2, and IRF9 (90), was able to bind to the ISRE in the *CR-C* region and to induce PD-1 expression (78).

1.6.4 Expression of PD-1 and its ligands

In the late 90s Nishimura *et al.* found that CD4−CD8− thymocytes express PD-1 at low levels during TCRβ rearrangement (91) and further showed that

Pdcd1$^{-/-}$ mice spontaneously developed autoimmune diseases demonstrating the important role of PD-1 in immune regulation (57, 92). Further studies also showed that PD-1 is only mildly expressed on resting T and B cells and that TCR and BCR ligation is necessary to induce PD-1 expression at the surface level (91, 93). Agata *et al.* described that mRNA transcription in mice does not strictly correlate with protein production and that PD-1 expression is sustained by the presence of persistent antigen stimulation (93).

PD-1 can be detected on T cells during chronic HIV infection where it negatively modulates expansion, cytokine production and cytolysis (94). Unlike CTLA-4, which is only expressed by T cells, PD-1 is broadly expressed on different cell phenotypes. PD-1 can be detected in a variety of cell phenotypes including CD4+ and CD8+ T cells, natural killer T (NKT) cells, natural killer (NK) cells, B cells, monocytes and dendritic cell (DC) subsets (25, 26, 95). PD-1 expression can also be induced by cytokine stimulation in absence of TCR-mediated stimulation. Cytokines that have roles in peripheral T cell expansion and in cell survival such as the γ-chain cytokines IL-2, IL-15, IL-7 and IL-21 have all been shown to induce PD-1 expression in human T cells *in vitro* (96).

CD4+ regulatory T cells are also able to express PD-1. In Treg cells PD-1 is thought to have a key role in the maintenance of Treg suppressive functions. Several lines of evidence from mice studies support this role. Wang *et al*. has shown that PD-L1 contributes to the conversion of naïve CD4+ T cells into CD4+ Treg cells (97). Similarly Francisco *et al.* demonstrated that PD-L1 synergizes with transforming growth factor beta (TGF-β) to promote induced Treg cell (iTreg) conversion and further showed that PD-L1–induced CD4+FoxP3+ Treg cells are able to suppress CD4+ Teff cells (98). Mechanistically, it appears that PD-L1 regulates Treg cell

development by crosslinking PD-1 on Treg cells and by antagonizing the Akt–mTOR signalling pathway. These findings are supported by experiments showing that PD-L1 deficiency in recombination-activating gene (Rag) mice impaired Treg cell conversion (98). Further evidence supporting the role of PD-1 for the correct function of Treg cells, comes from a study showing that anti-PD-1 drug nivolumab was able to overcome Treg-mediated suppression of CD8+ T cells by i) increasing resistance to Treg suppression and ii) by directly limiting Treg-suppressive capacity (99). Furthermore, in a different study nivolumab was shown to reverse Treg-mediated suppression of effector T cells restoring CD4+ T-responder cell proliferation and IFNγ production (100).

Similarly to PD-1, PD-L1 and PD-L2 are widely expressed across different cell phenotypes (25, 95, 101, 102). This differs from the distribution of the CTLA-4 ligands CD80 and CD86, which are mostly expressed by professional APCs and activated B and T cells (25, 26). PD-L1 can be detected on activated T and B cells, DCs, macrophages, mesenchymal stem cells and bone marrow–derived mast cells (103). PD-L1 is also expressed on a wide range of non-hematopoietic cells, in non-lymphoid tissues, and can be induced on parenchymal cells by inflammatory cytokines or tumorigenic signalling pathways. The PD-L1 ligand can also be found on different tumour cell types and in the tumour microenvironment on APCs where it is associated with an increased amount of tumour-infiltrating lymphocytes (TILs) and poorer prognosis (104-107). The second ligand for PD-1, PD-L2, has a higher affinity for PD-1 as compared to PD-L1 (108) but its expression patterns are still not extensively characterised. PD-L2 is primarily expressed on DCs and monocytes, but can be induced on other cells phenotypes, including T cells, and on tumour cells following

different stimuli from the local microenvironment (109). The expression of PD-L1 and PD-L2 is highly regulated by cytokines. Type 1 and type 2 interferons and TNFα induce PD-L1 expression in T cells, B cells, endothelial cells, and epithelial cells (95). The common γ chain cytokines IL-2, IL-7, and IL-15 are able to increase PD-L1 on human T cells and both PD-L1 and PD-L2 on monocytes (96). PD-L1 is also induced on monocytes by IL-10 and on CD19+ B cells by IL-21. IL-4 and granulocyte/macrophage colony-stimulating factor (GMCSF) have been shown to induce expression of PD-L2 on DCs (110-112).

1.7 Signalling through PD-1

PD-1 antagonises TCR-mediated signalling by modulating different pathways. Upon PD-1 engagement with its ligands, SHP-2 is recruited at the cytoplasmic tail of the receptor. This leads to a reduction in the phosphorylation of several TCR downstream signalling molecules including CD3ζ, zeta-associated protein of 70 kD (Zap70), and protein kinase C θ (PKC-θ) (70). Another key signalling target of PD-1 is the phosphatidylinositol-3-kinase (PI3K) pathway (36, 113). PD-1 blocks the activation cascade mediated by PI3K by reducing the phosphorylation of serine-threonine kinase Akt (36). Specifically, the PD-1-mediated inhibition of the PI3K–Akt signalling pathway involves the targeting of phosphatase and tensin homolog (PTEN) protein phosphorylation and phosphatase activity, which are mediated by CK2. PTEN is a serine–threonine phosphatase that regulates PI3K activity suppressing downstream signals of the PI3K complex. During T-cell activation, PTEN is phosphorylated by CK2 in the S380–T382–T383 serine-threonine cluster within the C-terminal regulatory domain (114, 115). This phosphorylation leads to a stabilized and more abundant PTEN but it also suppresses PTEN phosphatase activity (116).

PD-1 modulates this pathway by blocking CK2 thereby resulting in diminished PTEN abundance but increased PTEN phosphatase activity and consequent reduced signalling via PI3K and Akt (116).

Another mechanism by which PD-1 modulates T cell function is by targeting the Ras–MEK–ERK pathway (70, 113). In T cells, the protein PLC-γ1 is responsible for the formation of two molecules, diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 acts by releasing calcium from cell organelles, a mechanism important for T cell activation. Calcium and DAG activate a RasGRP1 protein, which is responsible for conversion of GDP-Ras into GTP-Ras leading to downstream activation of the MEK-ERK-MAP kinase pathway and to cell proliferation and cell differentiation (117). The PD-1 receptor acts by inhibiting the activation of PLC-γ1 impairing the activation of Ras and negatively modulating the MEK–ERK-MAP kinase pathway (113).

PD-1 can also induce the transcription factor BATF41, which is responsible for immune cell differentiation but is unable to modulate p38 MAP kinase activation (113) indicating that PD-1-mediated signalling does not cause a global inhibition but rather acts on specific pathways. Interestingly, T cell functions appear to be differentially sensitive to PD-1 expression. Wei *et al.* developed a model to study PD-1 signalling in primary human T cells and investigated how PD-1 expression affected T cell function. High levels of PD-1 expression were required to inhibit macrophage inflammatory protein 1 beta (MIP-1β) production while lower levels were required to block cytotoxicity and IFN-γ production, and very low levels of PD-1 expression were enough to inhibit TNFα and IL-2 production as well as T cell expansion (118).

Other studies have suggested that PD-1 might also have a role in modulating interactions between T cells and dendritic cells.

During T cell activation *in vivo*, the duration of contact between T cells and dendritic cells is highly variable and it ranges from minutes to several hours. Three distinct phases have been described for T cell priming. During phase I, lasting approximately 8 hours, T cells migrate and establish frequent contacts with dendritic cells. During phase II, which can range between 8 to 20 hours, T cells mainly form stable contacts with dendritic cells and initiate cytokine secretion. During phase III, which lasts for the subsequent 24 hours, T cells dissociate from dendritic cells and start to proliferate. T cells that remain in the migratory state instead of engaging with dendritic cells are thought to become anergic (119).

Fife *et al.* used a mouse model of diabetes in which islet-specific TCR transgenic T cells stimulated with an islet antigen peptide-mimic lose the natural stop signal becoming anergic and showed that blockade of PD-1 interaction with its ligand PD-L1 is able restores stable T cell–dendritic cell contacts preventing induction of anergy (120). Similarly, Honda *et al.* used a mouse model of hypersensitivity in the skin to demonstrate that blockade of PD-1/PD-L1 enhances T cells interactions with antigen-bearing APCs (121). These two studies suggest that PD-1 might prevent the formation of stable interactions between T cells and dendritic cell reducing the development of effector functions and reducing APC-mediated activation. In contrast with these findings, it was shown that in a model of lymphocytic choriomeningitis virus infection, PD-1 blockade during the early stage of infection led to rapid detachment and migration of T cells away from antigen-bearing APCs (122). The observed differences are likely a consequence of the different experimental models used in these studies but reveal the multiple levels at which this pathway can affect the immune response.

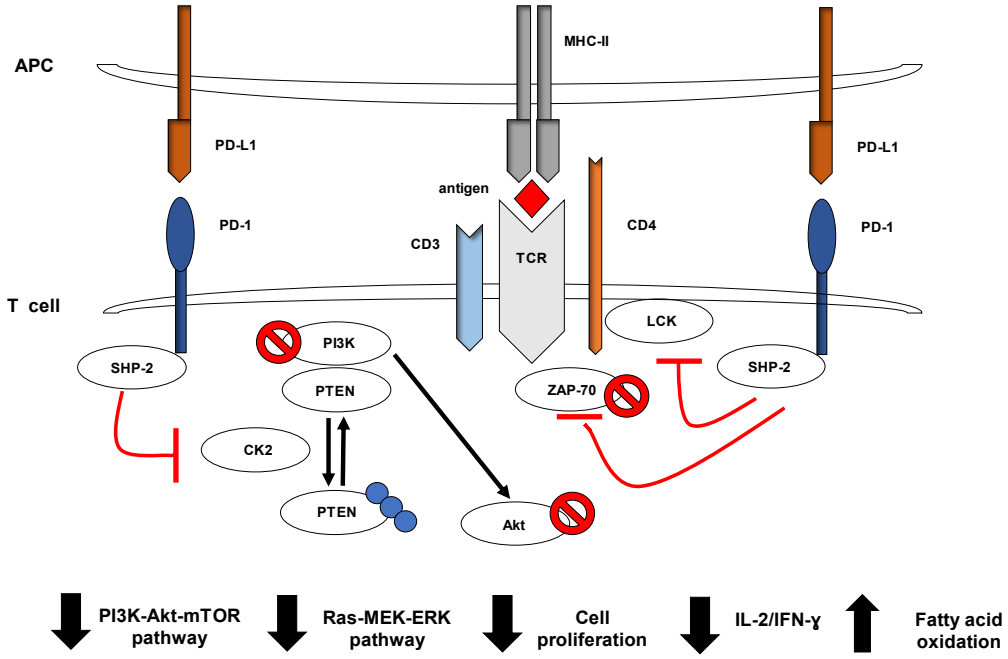

Figure 1.4 Effects of PD-1 on different signalling pathways in T cells.
During T-cell receptor (TCR) crosslinking the cytoplasmic tail of PD-1 become phosphorylated. SHP-2 is recruited to the ITSM. Phosphorylation of TCR proximal signalling molecules, including LCK and ZAP-70, is impaired. Activation of the PI3K–Akt–mTOR and Ras-MEK-ERK pathway is also inhibited. These events modulate cell-cycle progression, gene transcription, metabolism, and epigenetic programs in T cells.

1.8 PD-1 effect on T cell metabolism

The metabolism of resting T cells is characterised by oxidation of glucose-derived pyruvate, along with lipids and amino acids. This process produces adenosine triphosphate (ATP), which is required for immune surveillance. Following activation, T cells undergo metabolic reprogramming and aerobic glycolysis and glutamine oxidation intensifies. These mechanisms lead to production of biosynthetic precursors required for rapid cell growth and proliferation. Once the immune response is completed, the surviving cells acquire a memory phenotype and revert back to lipid oxidation (123). Patsoukis *et al.* have demonstrated that PD-1 engagement in activated T cells suppresses oxygen consumption and T cells become unable to engage in glycolysis and glutaminolysis. Conversely, the rate of fatty acid β-oxidation (FAO) is

increased (124). PD-1 modulates cell metabolism by promoting FAO of endogenous lipids. This is achieved by increased expression of carnitine palmitoyl transferase and induction of lipolysis as shown by increased adipose triglyceride lipase, increased glycerol and elevated release of fatty acids. Upon PD-1 engagement, glutathione, a molecule with strong antioxidant properties is decreased indicating the presence of an oxidative environment in T cells receiving PD-1 signals (124). The possibility that PD-1 may lead to a more oxidative environment is further suggested by a study showing that after allogeneic bone marrow transplantation, both murine and human alloreactive T cells upregulate PD-1 and increase the production of reactive oxygen species (ROS) derived from fatty acid oxidation facilitating apoptosis of alloreactive T cells. Furthermore, cell sensitivity to metabolic inhibition by F1F0–ATP synthase complex inhibitors also increase suggesting that PD-1-mediated signalling is able to skew the cell towards an oxidative environment (125). These data suggest that the effect on T cell metabolism upon PD-1 engagement might have a role in PD-1–mediated T-cell dysfunction during chronic infections and cancer.

1.9 Reverse signalling via PD-L1 & PD-L1:CD80 interaction

PD-L1 and PD-L2 ligands modulate TCR and BCR signalling by engaging with the PD-1 receptor but new evidence indicate that they might also deliver reverse signals on APCs. Kuipers *et al.* cultured bone marrow-derived mouse DCs with a soluble PD-1-Ig fusion protein containing the extracellular domain of mouse PD-1 fused to the constant region of human IgG and demonstrated that this soluble form of PD-1 (sPD-1) inhibited DC activation/maturation and increased IL-10 production in a mechanism 2,3-dioxygenase (IDO)-independent. Neutralisation of sPD-1 with anti-PD-1 was shown to prevent these effects, suggesting a PD-1-specific effect occurring

via PD-L1 or PD-L2 although the specific ligand responsible for such an effect was not investigated (126). Different lines of evidence also indicate that B7-1 (CD80) can bind and signal via the PD-L1 ligand and vice versa. Using the technique of surface plasmon resonance Butte *et al.* (127) demonstrated that there is a specific interaction between PD-L1 and B7-1 (127). The PD-L1:B7-1 interaction was found to have an affinity (~1.7 μM) which is intermediate between B7-1:CD28 (4 μM), B7-1:CTLA-4 (0.2 μM) and PD-L1:PD:1 (0.5 μM) affinities. The authors found that B7-1:PD-L1 interactions induced an inhibitory signal into mouse T cells. Specifically, ligation of PD-L1 on CD4+ T cells by B7-1 but also ligation of B7-1 on CD4+ T cells by PD-L1, was shown to deliver an inhibitory signal. Interestingly, anti-CD3/B7-1-coated beads were able to reduce *in vitro* proliferation of CD28/CTLA4$^{-/-}$ T cells while no effect on proliferation and cytokine production was found on T cells lacking all the receptors for B7-1 (CD28, CTLA-4, and PD-L1).

This indicates that B7-1 acts specifically through PD-L1 on the T cell in the absence of CD28 and CTLA-4. Similarly, anti-CD3/PD-L1-coated beads were able to decrease proliferation and cytokine production in T cells lacking the receptor for PD-L1 (PD-1) while proliferation was no longer affected in T cells lacking all known PD-L1 receptors (PD-1 and B7-1) demonstrating the inhibitory effect of PD-L1 ligation of B7-1 on T cells. These results are important as they demonstrate a specific bidirectional interaction between B7-1 and PD-L1 that inhibits T cell responses (127).

Very little is known about PD-L1 function on PD-L1 expressing T cells. Latchman *et al.* generated a PD-L1-deficient mouse and showed that CD4+ and CD8+ T cell responses were markedly enhanced as compared to wild-type mice both *in vitro* and *in vivo*. Compared to wild-type dendritic cells, PD-L1$^{-/-}$ dendritic cells were able to stimulate to a greater extent CD4+ T cell responses and PD-L1$^{-/-}$ CD4+ T cells were

shown to produce more cytokines than wild-type CD4+ T cells *in vitro*, indicating an inhibitory role for PD-L1 on both APCs and T cells (102).

1.10 Immune checkpoints: TIGIT, TIM-3 and LAG-3

CTLA-4 and PD-1 are strong regulators of T cell responses and effective targets in cancer immunotherapy. Furthermore, CTLA-4-Ig (abatacept) has proven successful in the treatment of chronic autoimmune diseases such as RA and JIA. Unfortunately, many cancer and arthritis patients fail to respond to therapies based on immune checkpoints regulatory activity. The next sections will briefly describe three inhibitory receptors, TIGIT, TIM-3 and LAG-3, which have been shown to modulate autoimmunity but also cancer progression. These receptors are promising targets for future clinical trials and a better understanding of their functions will be important to introduce to the clinic new therapies that target these receptors.

1.10.1 TIGIT

The T cell immunoreceptor with Ig and ITIM domains (TIGIT) is an inhibitory receptor of the immunoglobulin (Ig) superfamily. TIGIT is a type 1 transmembrane protein containing an IgV extracellular domain and an immunoglobulin tail tyrosine (ITT)-like phosphorylation motif followed by an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail. TIGIT ligands are Ig-like transmembrane cell adhesion molecules called nectins and include CD155, CD112 and CD113 (128). TIGIT is not found on naïve T cells, but is expressed on activated and memory T cells, Treg cells (128) and on NK cells and NKT cells (129) in both mice and humans. In human, TIGIT crosslinking with CD155 induces a tyrosine phosphorylation on the receptor's ITT domain followed by the recruitment of the

phosphatase SHIP-1 leading to inhibition of phosphatidylinositol 3-kinase (PI3K), MAPK, and NF-kB signalling (130). TIGIT is also able to downregulate NK cell killing ability and to induce production of anti-inflammatory IL-10 by APCs (131).

A single nucleotide polymorphism (SNP) in the positive regulator CD226 (Gly307Ser) of the TIGIT-CD226 pathway is associated with predisposition to multiple autoimmune diseases in humans including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis (132). In the in collagen-induced arthritis (CIA) models blockade of TIGIT is associated with exacerbation of the disease and with enhanced proinflammatory T cell responses (133) suggesting that TIGIT plays an important role in maintaining peripheral tolerance by negatively modulating T cell activation. In a pre-clinical murine model, Johnston *et al.* demonstrated that TIGIT is co-expressed with PD-1 in tumour-infiltrating CD8+ T cells and that blockade of both PD-L1 and TIGIT leads to a restored anti-tumour immunity which is higher as compared to PD-L1 blockade alone (134). In human melanoma patients, Chauvin *et al.* showed that TIGIT and PD-1 are overexpressed on both tumour antigen-specific CD8+ T cells and tumour-infiltrating CD8+ T cells and that blockade of these receptors leads to increased cell proliferation, cytokine production, and degranulation of cytotoxic CD8+ T cells (135). Collectively, these data indicate that TIGIT acts by dampening effector T cell responses and promotes T cell regulation.

1.10.2 TIM-3

The T cell/transmembrane, immunoglobulin, and mucin 3 (TIM-3) co-inhibitory receptor is a transmembrane proteins containing a single IgV domain followed by a mucin domain and a cytoplasmic tail with a tyrosine-based signalling motif (136). TIM-3 is expressed by a subset of activated CD4+ T cells and it is further

upregulated upon anti-CD3/CD28 stimulation. TIM-3 is expressed at high levels on *in vitro* polarized Th1 cells and at lower levels on Th17 cells (137). TIM-3 expression is also upregulated by antigenic stimulation and in response to proinflammatory cytokines. TIM-3 interaction with its ligand Galectin-9 (Gal-9) causes an inhibitory signal resulting in apoptosis of Th1 cells (138, 139) and cytotoxic CD8+ T cells *in vitro* (140). Blockade of Gal-9 via siRNA and Gal-9 KO in mice (138) induces symptoms of experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (141). Furthermore, both TIM-3 blocking antibody and TIM-3–Ig fusion protein have been shown to worsen the symptoms of EAE (138, 142, 143) and type I diabetes in non-obese (NOD) mice (144). In line with experiments performed on TIGIT, it has been shown that in mice, combined treatment with anti–Tim-3 and anti–PD-L1 enhances antitumor immune responses as compared to single treatment indicating that both pathways are important in tumour clearance (145). These data strongly support the idea that TIM-3 on activated T cells plays a critical role in attenuating immune responses.

1.10.3 LAG-3

LAG-3 is another T-cell regulatory molecule belonging to the Ig superfamily. LAG-3 associates with the TCR:CD3 complex following TCR engagement and negatively regulates signal transduction (146). This molecule can be found on activated CD4+ and CD8+ T cells and on activated CD4+ Tregs (147). LAG-3 is also detected on Tr1 cells (148) on a subset of NK cells (149), B cells (150) and plasmacytoid DCs (151). In non-activated T cells, Bae *et al.* and Woo *et al.* have shown that LAG-3 is localized and degraded within the lysosomal compartments (152, 153). LAG-3 is connected to development of autoimmunity in mice. LAG-3 blockade

or LAG-3 deficiency has been shown to accelerate diabetes in the NOD mice (154, 155), while combined LAG-3 and PD-1 deficiency in mouse is a trigger for the development of severe autoimmune conditions and early death in several different genetic backgrounds (154, 156) indicating that LAG-3 is a strong negative T cell regulator. LAG-3 and PD-1 can be found on both CD4+ and CD8+ tumour infiltrating lymphocytes in several pre-clinical murine models of cancer and blockade of both the Lag-3 and PD-1 pathways has been shown to ameliorate anti-tumour responses mediated by CD8+ T cells (156). Similarly to mouse, co-expression of Lag-3 and PD-1 can be found in dysfunctional CD8+ T cells in human ovarian cancer and, as observed in pre-clinical cancer models, Lag-3 and PD-1 co-blockade led to higher cytokine production and proliferation of CD8+ T cells (157). Indications of the potential of LAG-3 to regulate immune responses come from the observation that soluble Lag-3-Ig is able to modulate the reduce tumours growth in mice (158) and in humans (159).

1.11 The role of PD-1 in cancer

The role of PD-1 in regulating T cell activation in human autoimmune diseases remains unclear. On the contrary, PD-1 function has been extensively investigated in cancer studies. Hence, it is important to illustrate the current literature on this matter.

The immune system has the ability to recognise and eliminate malignant cells. However, the tumour microenvironment is highly immunosuppressive and tumour cells have developed mechanisms to evade immune regulation. T cell inhibitory pathways such as the PD-1- and CTLA-4-mediated pathways are in place to regulate the strength and duration of immune responses. Through these pathways the immune system is able to limit immune-mediated tissue damage, control inflammation and maintain tolerance to prevent autoimmunity.

A key component of the immunosuppressive milieu found in many tumours is the expression of PD-L1 on malignant cells. Early studies performed in animal models demonstrated that expression of PD-L1 on tumours inhibits T cell activation and reduces lysis of malignant cells leading to increased death of tumour-specific T cells (160, 161). Importantly, disrupting the PD-1/PD-L1 pathway by using anti-PD-L1 monoclonal antibodies or by injecting tumour cells into *Pdcd1*$^{-/-}$ mice was shown to augment antitumor responses and tumour-specific cytotoxicity (161-163). Tumour-associated APCs can also modulate immune responses via the PD-1 pathway. Curiel *et al.* demonstrated that tumour environmental factors can induce PD-L1 expression on a population of tumour-associated myeloid DCs, and further showed that T cells cultured with PD-L1-blocked DCs had a stronger ability to inhibit autologous human ovarian carcinoma growth in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (164). This indicates that upregulation of PD-L1 in the tumour

microenvironment and on tumour infiltrating APCs have a role in reducing T-cell cytotoxic activity.

In human, PD-L1 expression has been demonstrated on a wide variety of tumours including, but not limited to, lung, ovarian, melanoma, bladder, pancreas, gastric, non–small cell lung cancer, breast cancer, squamous cell head and neck cancer and Hodgkin lymphoma. It is now widely accepted that cancer cells use this ligand to negatively modulate the cytotoxic activity of tumour-specific PD-1+ T cells (165-176). Multiple mechanisms drive PD-L1 and PD-L2 expression on tumour cells. These mechanisms include constitutive activation of signalling pathways, chromosomal copy gain, viral infection and cytokine stimulation. Persistent activation of different signalling pathways including the epidermal growth factor receptor (EGFR) pathway, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–Akt pathway (177-180) can increase PD-L1 expression in animal model systems. Elevated expression of signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor (HIF)-1 transcription factors (181, 182) are also associated with PD-L1 upregulation in various cancer types through both transcriptional and post-transcriptional mechanisms.

Chromosomal copy gain is another identified mechanism driving PD-L1 upregulation. In classical Hodgkin's lymphoma, a type of cancer characterised by the presence of a specific malignant cell type called Reed-Stemberg Cell (183), copy number alterations involving chromosome 9p24.1 result in overexpression of the PD-1 ligands PD-L1 and PD-L2 on tumour cell surface and it is associated with poor prognosis (174-176). Copy alterations in JAK2, which is also located on chromosome 9p24.1, lead to increased JAK-STAT signalling and further PD-L1 overexpression

(175). PD-L1 expression on tumour cells is also modulated by viral infection, as shown by the presence of positive PD-L1 cells in the Epstein–Barr virus (EBV)–positive gastric and nasopharyngeal cancers (184, 185), and by epigenetic mechanisms through microRNAs (miRs). MiRs are non-coding single-stranded RNAs of 22–24 nucleotides in length, which have the ability to regulate gene expression by binding target gene mRNAs preventing translation into protein (186). Several lines of evidence show that miR-513, miR-570, miR-34a and miR-200 all have an inverse correlation with PD-L1 expression complementing with three untranslated regions of PD-L1 mRNA and repressing PD-L1 protein expression (187-190).

Malignant cells can also upregulate PD-L1 in response to a proinflammatory milieu. When tumour-infiltrating T cells recognise tumour antigens presented via MHC molecule by APCs they mount tumour-specific responses, which include production of cytokines. Within the tumour microenvironment IFN-γ and IL-4 have been shown to induce expression of PD-L1 and PD-L2 on cancer cells (107). Inflammatory mediators can also induce expression of PD-L1 and PD-L2 on other cell types in the tumour microenvironment including macrophages, dendritic cells, and stromal cells (107). Activated PD-1+ tumour-infiltrating T cells engage with PD-L1+ and PD-L2+ cells and this results in a very selective suppression of tumour-specific T cells activity.

1.12 The other side of immune checkpoint blockade: inflammation

In recent years, drugs blocking the negative immune checkpoint regulators CTLA-4 and PD-1 have shown remarkable clinical activity. These therapies have added immeasurably to the longevity of cancer patients. However, they come with an associated cost: the development of numerous inflammatory conditions that can resemble autoimmune responses. The following sections will describe the role of PD-1 in the development of autoimmunity and will review the current literature related to the PD-1 pathway in rheumatoid arthritis (RA) and psoriatic arthritis (PsA). Next, a description of RA and PsA will be provided along with data outlining differences and similarities between the two diseases, leading up to the thesis rationale.

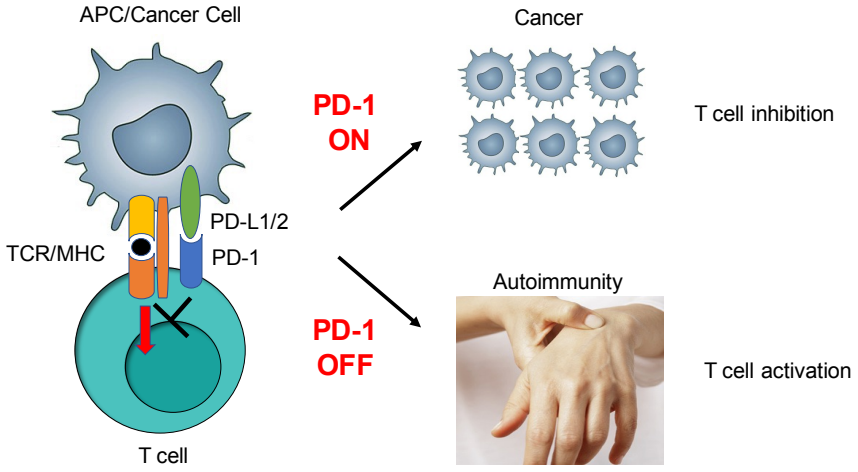

Figure 1.5 Clinical application for PD-1 agonists and antagonists.
Signalling via PD-1 (ON) leads to cytotoxic T cell inhibition and tumour progression. PD-1 antagonists that enhance T cell-mediated immune response by blocking PD-1 are successful in cancer treatment. Conversely, a lack of signalling (OFF) via PD-1 might be responsible for hyperactivation of T cells and autoimmunity. PD-1 agonists that enhance PD-1-mediated signalling may be useful in treating autoimmune diseases including rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

1.12.1 PD-1 and CTLA-4 blockade in human cancer

In multiple preclinical models, blockade of the PD-1/PD-L1 pathway enhanced antitumor T cell cytotoxicity and proliferation, thereby promoting more efficient tumour clearance (191). PD-1 is now used as a therapeutic target in a vast array of cancers and targeting the PD-1 pathway has been shown to drive expansion of oligo-clonal populations of tumour-infiltrating CD8+ T cells (191, 192). Checkpoint inhibitor drugs directed towards the PD-1 pathway such as nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), atezolizumab (anti-PD-L1) and towards CTLA-4 (ipilimumab), have shown remarkable clinical activity in various tumours (165, 193-196). Given the success of these drugs in treating cancer, two new compounds named avelumab and durvalumab (both anti-PD-L1 mAb) have recently received commercialisation approval and are now available for cancer patients. These therapies are ground breaking in enhancing disease free survival and life expectancy (165). However, because the PD-1 and CTLA-4 checkpoint pathways have also key roles in the maintenance of self-tolerance, blocking their biological activity can lead to the development of serious adverse events (AEs) including autoimmune-like inflammatory conditions (197, 198). The strong immune responses following checkpoint inhibitor therapy can cause damage to tissues and organs that are normally protected by the correct functional activity of the PD-1 and CTLA-4 pathways. Immune-related AEs can include mucosal and cutaneous toxicity, gastrointestinal toxicity, pulmonary, endocrine and renal toxicity and in some cases life-threatening disorders such as Sweet's syndrome, Stevens-Johnson syndrome and toxic epidermal necrolysis (199-202). Dermatological AEs, have been described in >30% of patients who received nivolumab or pembrolizumab for melanoma (202) while 15% of patients treated with nivolumab and ipilimumab combination therapy developed

hypothyroidism (203). More severe AEs like colitis, pneumonitis and hepatitis although less common were reported in patients with melanoma treated with ipilimumab (54, 203). In non-small cell lung cancer, 5% of patients treated with anti-PD-1 (nivolumab) developed pneumonitis (195). Pneumonitis was also seen in patients treated for Renal Cell Carcinoma (204) and in melanoma patients without BRAF mutation (194). Finally, autoimmune hepatitis was reported in 5%–10% of patients treated with either nivolumab or ipilimumab (194, 205). Treatments targeting PD-1 have shown a more favourable safety profile compared to those targeting CTLA-4, with lower toxicity and milder inflammatory side effects (199, 206). This is in line with the milder inflammatory pathology observed in PD-1-deficient compared to CTLA-4-deficient mice and likely reflects the more restricted role of PD-1 in constraining on-going immune responses in contrast to the role of CTLA-4 in immune homeostasis (95, 207).

1.12.2 PD-1 and autoimmunity in mice

The fact that checkpoint inhibitor therapies are associated with recurrent AEs and autoimmune-like conditions should not be unexpected. Some of the observed side effects following PD-1 inhibitor therapy in cancer patients, such as renal or endocrine toxic effects and cardiomyopathy, closely resemble the autoimmune manifestations described in PD-1–deficient mice and in *in vitro* targeting of the pathway via PD-1 blockade (57, 58, 202, 208). Further studies on autoimmune disease models have shown that PD-1 interactions with its ligand PD-L1 are important during the initial phase of activation and expansion of self-reactive T cells and during the process of antigen re-encounter. In the non-obese diabetic (NOD) mouse model of autoimmune T cell–mediated diabetes, PD-L1 is increased in the pancreas islet cells and loss or

blockade of PD-1 or PD-L1 can exacerbate diabetes, insulinitis and induce proinflammatory cytokine production by T cells (110, 209, 210). Further studies have also shown that PD-L1 expression on non–bone marrow–derived cells, including islet cells, inhibits T cell effector function in tissues (211) suggesting an important role for the PD-1 pathway in controlling immune-mediated tissue damage in the periphery (212). The PD-1 pathway is also important in controlling self-reactive T cells in the experimental autoimmune encephalomyelitis (EAE) model of human multiple sclerosis (MS) and PD-1, PD-L1 and PD-L2 are increased on cellular infiltrates within the meninges during active EAE disease in C57BL/6 mice (110). Salama *et al.* showed that treatment with anti-PD-1 or anti-PD-L2 blocking antibodies during the induction of EAE was able to exacerbate disease severity leading to increases in reactive T cells (213).

Interestingly, further evidence suggest that the B7-1:PD-L1 axis might also have a role in controlling the development of autoimmunity *in vivo,* albeit with a weaker impact that the classic PD-1:PD-L1 mediated mechanism. Yadav *et al.* have shown that in the NOD mouse model, $Cd80^{-/-}$ NOD mice develop autoimmunity more rapidly as compared to classical NOD but that in $Pdcd1^{-/-}$ and $Cd274^{-/-}$ NOD mice stronger inflammation is found as compared to the $Cd80^{-/-}$ NOD (214). This suggests a weaker effect mediated by the B7-1:PD-L1 axis as compared to the PD-1:PD-L1 interaction. Further studies are needed to clarify the role of PDL1:B7-1 interactions in promoting peripheral tolerance.

1.12.3 PD-1 in mouse and human arthritis

A strong indication that PD-1-mediated signalling is important in rheumatic disease comes from studies on the collagen II induced arthritis (CIA) murine model. The CIA is a murine experimental model of human RA, where intradermal administration of type II collagen leads to joint swelling, articular cartilage destruction and ankylosis of the joints. Using this model, Wang *et al.* showed that triggering the PD-1 receptor in CIA mice through administration of a PD-L1 immunoglobulin ameliorated the disease as assessed by clinical arthritis score and histology of the joint assessments. Furthermore, it was shown that PD-L1-Ig treatment reduced the levels of proinflammatory cytokines IL-17 and IL-23 in the serum of treated mice and inhibited CII-specific splenocyte proliferation (215). Similarly, Raptopoulou *et al.* demonstrated that *Pdcd1*$^{-/-}$ mice had increased incidence/severity of CIA, enhanced T cell proliferation and increased production of IFN-γ and IL-17 cytokines. In line with work from Wang *et al.* (215) it was shown that PD-L1 administration ameliorated the severity of CIA and reduced T cell responses (216). More recently, Yang *et al.* confirmed that PD-1 deficiency leads to the development of severe CIA in mice and described aberrant antigen-specific Th17 responses and deregulated activation of PKC-θ and Akt. Interestingly, it was shown that treating *Pdcd1*$^{-/-}$ mice with an inhibitor of PI3K, which is upstream of PKC-θ and Akt, significantly suppressed the disease severity (217).

In humans, indications that disruption of the PD-1 pathway might lead to development of autoimmunity are found in studies showing that polymorphisms in the *PDCD1* gene are associated with different human autoimmune diseases, including SLE, type 1 diabetes, RA, psoriasis, ankylosing spondylitis (AS) and MS (218, 219). In line with these findings, a growing spectrum of rheumatic events is emerging

following PD-1 inhibitor therapies in cancer patients. Arthritis and tenosynovitis have been described in metastatic melanoma patients treated with anti-PD1 antibody (220). Man *et al*. reported a case of psoriasis and psoriatic arthritis induced by anti-PD-1 nivolumab in a patient with advanced lung cancer (221). More recently, Cappelli *et al.* analysed 13 patients treated with anti-CTLA-4 or a combination of anti-CTLA-4 and anti-PD-1 and found that 9 of 13 patients developed inflammatory arthritis with cases of synovitis and inflammatory synovial fluid. In the studied cohort further AEs included pneumonitis, colitis, interstitial nephritis and thyroiditis and patients required treatment with corticosteroids or in some cases methotrexate (MTX) and antitumor necrosis factor (anti-TNF) therapy (222).

Due to the lack of dedicated studies, it is unclear whether i) pre-existing autoimmune disorders are exacerbated by checkpoint inhibitor therapy and ii) whether the presence of AEs augments or weakens anti-tumour responses. A recent study performed on advanced melanoma patients with pre-existing autoimmune disorders including RA, psoriasis, inflammatory bowel disease, multiple sclerosis, autoimmune thyroiditis and SLE found that CTLA-4 blockade with ipilimumab was associated with exacerbations of autoimmune disease in 27% of analysed patients (223). Currently, it remains unclear whether anti-PD-1 blockade is able to exacerbate pre-existing autoimmunity. These preliminary results show that immuno-oncology therapies and development of autoimmune-like AEs are strongly interconnected and indicate that comprehensive studies are required to properly understand the biological mechanisms involved in checkpoint inhibitors treatment. Further characterisation of PD-1 in human arthritis is provided in chapter 3, chapter 4 and chapter 5.

1.13 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that typically affects the hands and feet, although any joint lined by a synovial membrane may be affected. RA is characterised by cell migration to the synovial compartment, synovial and systemic inflammation, autoantibody production (i.e. rheumatoid factor and anti-citrullinated protein antibody (ACPA), cartilage and bone destruction and systemic features, which include cardiovascular, pulmonary and skeletal disorders (224). RA affects 23.7 million people worldwide and although multiple treatments are available, up to one third of patients do not adequately respond, and about half stop responding to any particular disease-modifying antirheumatic drug (DMARD) within five years (225-230). As a result, additional investigations to develop alternative treatments are needed.

1.13.1 Genetics and environmental factors in RA

It has been demonstrated that in patients who are positive for rheumatoid factor (RF) or ACPA there is a strong association with the human leukocyte antigen (HLA)-DRB1 locus and that alleles containing a common amino acid motif (QKRRA) in the HLA-DRB1 locus confer susceptibility to RA (231). This suggests that RA is a disease where predisposing T cell repertoire selection, antigen presentation or alterations in peptide affinity might have a role in promoting autoreactive immune responses. Other identified risk factors associated with predisposition to ACPA-positive RA include dysregulation in nuclear factor κB (NF-κB)–dependent signalling, T cell stimulation, T cell activation and functional differentiation (i.e. TRAF1-C5, STAT4, PTPN22, CTLA-4 and PD-1) (232-236). Genetic risks for ACPA-negative RA are also important and include different HLA alleles (HLA-DRB1*03), interferon

regulatory factors (Interferon response factor 5) and lectin-binding proteins (237). Studies on gene and environment interactions have shown that blood transfusions, obesity and especially smoking and other pulmonary stress like silica exposure, can increase the risk of developing RA especially among individuals with susceptibility HLA-DR4 alleles (238). Infectious agents such as Epstein-Barr virus, cytomegalovirus, *Escherichia Coli* and *Porphyromonas gingivalis* have also been linked to the development of RA (239-241).

1.13.2 Innate immunity in RA

Different innate effector cells can be found in RA. Macrophages, mast cells, natural killer cells and neutrophils can be detected both in the synovial membrane and in the synovial fluid (242, 243). Macrophages are the central effectors in promoting inflammation in RA. Macrophages are activated by toll-like receptors (TLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (244), inflammatory cytokines, T cell interaction and by the protease-rich milieu found in the inflamed synovium (245). Monocytes play several different roles in RA including, but not limited to, antigen presentation to T cells, polarisation of CD4+ T helper cells, recruitment of T helper cells in the arthritic joint and modulation of CD4+ Treg cells function through production of soluble mediators (246). Monocytes can release proinflammatory cytokines including TNFα, IL-1β, IL-6, IL-15, IL-12 and IL-23 and they are able to produce reactive oxygen species (ROS) and matrix degrading-enzymes (246-248). RA-derived monocytes from the site of inflammation are able to specifically promote Th17 responses in a TNFα and IL-1β, but not IL-6 or IL-23-dependent manner, compared with resting CD14+ monocytes from the blood (13). CD14+CD16- and CD14+CD16+ monocytes activated *in vitro* with LPS or with

cytokines known to be present in the RA joint, can induce expression of proinflammatory cytokines IL-17 and IFNγ by CD4+CD25+CD127^{low} Treg cells, a mechanism that occurs in a IL-6-, TNFα-, and IL-1β-dependent manner (249). Recent work from our lab also showed that SF-derived monocytes from RA patients are resistant to spontaneous and anti-Fas-mediated apoptosis, they display increased levels of mature mir-155 expression and a corresponding decrease of two mir-155-target mRNAs, namely apoptosis mediators CASP10 and APAF1, suggesting that an increase in mir-155 may partially contribute to this apoptosis-resistant phenotype (250).

Mast cells and neutrophils are also highly represented in the inflamed synovium and synovial fluid. Mast cells can produce vasoactive amines, cytokines including IL-17A, chemokines and proteases while neutrophils contribute to inflammation by synthesizing prostaglandin, proteases and reactive oxygen intermediates (251-253). NK cells are another cell phenotype detected in the synovial fluid of RA patients. RA-derived NK cells are generally CD56^{bright}, highly CD94/NKG2A positive and have decreased expression of KIRs and CD16. SF-derived NK have been shown to produce more TNFα as well as IFNγ compared to peripheral blood derived NK cells and have upregulated expression of several chemokine receptors and adhesion molecules suggesting a role in cell recruitment into the inflamed synovium (254-256).

1.13.3 Adaptive immunity in RA

The genetics of RA and the presence of autoantibodies indicate that adaptive immunity is at the centre of RA pathogenesis. The inflamed RA synovium contains both lymphoid (T, B and NK cells) and myeloid cells such as monocytes,

macrophages and dendritic cells. Both cell lineages are capable of producing proinflammatory cytokines contributing to a state of chronic inflammation (257). Myeloid cells express HLA class II molecules and a variety of costimulatory molecules important for T cell activation and antigen presentation (258-260). B lymphocytes have multiple important functions in RA. They are responsible for the production of the rheumatoid factors and anti-citrullinated protein antibodies, they present antigens to T cells and they further activate them through expression of costimulatory molecules. B cells are able to produce chemokines and cytokines promoting leukocyte infiltration into the joints and can contribute to angiogenesis and synovial hyperplasia (261, 262).

The pathogenic role of CD20+ B cells is confirmed by the efficacy of cell-depleting drug rituximab which is approved for treatment in RA (263). RA has been long considered to be a type 1 helper T cells (Th1)-mediated disease. However, in recent years, another cell subset named type 17 helper T cells (Th17) has become increasingly important in the context of RA progression. Macrophage- and dendritic cell-derived cytokines such as transforming growth factor β (TGF-β), IL-1β, IL-6, IL-21, and IL-23 can all promote Th17 differentiation. The Th17 subset produces interleukin (IL)-17A, IL-17F, IL-21, IL-22, IL-6 and TNFα and it positively correlates with systemic disease activity (264-266).

1.13.4 Key cytokines & intracellular signalling pathways in RA

The cytokine produced by the numerous SF-derived populations are central in the pathogenesis of RA and their pattern of expression appears to change over time. Raza *et al.* analysed the cytokine profile of newly diagnosed early RA patients and patients with established RA and described that in early arthritis IL-2, IL-4, IL-13, IL-

17, IL-15 were significantly elevated as compared to patients who did not develop RA. Furthermore, this specific cytokine profile was no longer present in established RA suggesting that certain cytokines might be involved in influencing the microenvironment required for chronic RA (267). Cytokines TNFα, IL-6 and IL-1β play a fundamental role in the progression of RA (257, 268). TNFα drives cytokine and chemokine expression and it has been shown to upregulate endothelial-cell adhesion molecules promoting cell migration. TNFα is also important for fibroblast function and is a potent inducer of angiogenesis. Finally, TNFα-mediated signalling is capable of suppressing regulatory T cells promoting effector T cell proliferation (269-272). IL-6 can drive lymphocyte activation, autoantibody production and promote acute phase responses and lipid-metabolism dysregulation. (270, 273). Interleukin-1 family cytokines like IL-1α and IL-1β are also found in RA (274). IL-1β is a potent proinflammatory cytokine. It promotes activation of leukocytes, endothelial cells, chondrocytes and osteoclasts (257). Furthermore, IL-1β is able to trigger vasodilatation and to attract monocytes and neutrophils at the site of inflammation (270, 275). The central role of these cytokines has been confirmed by successful therapeutic blockade of TNFα and IL-6 in patients with RA (276).

Although the network of intracellular signalling pathways that regulate cytokine-receptor mediated functions in RA is highly complex, different attempts have been made to target new molecules. The JAK pathway mediates the function of several cytokines, interferons and growth factors and it is upregulated in RA (277). Unlike other therapies for RA, which are directed at extracellular targets such as pro-inflammatory cytokines, novel approaches targeting the intracellular pathways modulated by inflammatory cytokine networks have shown promising results. Recent progress in treating RA has been achieved with Janus kinase (JAK) inhibitors

(Jakinibs) (278, 279) and this pathway is now targeted in RA by a dedicated drug (tofacitinib) (280, 281). Interestingly, the use of this JAK1/JAK3 inhibitor has shown promising results in other inflammatory diseases including psoriasis (282, 283) and psoriatic arthritis (284, 285) indicating that targeting intracellular signalling pathways might be beneficial in group of patients which do not respond to or progress after currently available treatments.

Another molecule that has drawn interest as a new possible target for treatment was the spleen tyrosine kinase (Syk). Syk is a cytoplasmic tyrosine kinase that is an important mediator of immunoreceptor signalling in a variety of cells, including mast cells, macrophages, neutrophils, and B cells (286). Syk expression can been detected in RA synovium and increased levels of phosphorylated Syk have been described in RA synovial tissue as compared to osteoarthritis controls. Syk activation is important for TNF–induced cytokine and metalloproteinase production in RA synoviocytes (286). R788 (fostamatinib), a prodrug of active metabolite R406, was shown to inhibit Syk kinase and to be active in a variety of *in vitro* and *in vivo* models, suggesting potential activity in the treatment of RA. Fostamatinib was investigated as a new therapy in rheumatoid arthritis (287-289) but unfortunately limited efficacy as compared to placebo in a Phase III study lead to dismissal of it (290). Other intracellular targets, including PI3K, MAPK, Bruton's tyrosine kinase and components of the NF-κB pathway might offer opportunities for new therapeutic strategies (291).

1.14 Psoriatic Arthritis

Psoriatic arthritis (PsA) is an inflammatory disease of the joints that develops in up to 30% of patients with a skin condition called psoriasis. It is an asymmetric arthropathy, which causes pain, stiffness of the joints and an overall reduction of the quality of life. Psoriasis usually precedes the development of arthritis by an average of 10 years, although in some cases the two diseases might appear simultaneously (292, 293). The diagnosis of PsA is based mainly on clinical and imaging analysis since no specific biomarkers are currently known. In 50% of PsA patients, the disease affects the distal joints. This is not observed in RA. During disease onset, PsA is asymmetrical but with time can develop as polyarticular and symmetrical (294).

1.14.1 Genetic and environmental factors in PsA

PsA is a highly heritable disease (295). PsA is associated with class I MHC alleles. Frequencies of HLA-B*08 B*27, B*38, and B*39 have all been observed (40). Genome wide association analyses have shown that there is an association between PsA and polymorphisms in the gene encoding for the IL-23 receptor (IL23R). Furthermore, variants in nuclear factor κB (NF-κB) gene expression (TNIP1) and signalling (TNFAIP3) are also associated with psoriatic arthritis. Association studies have identified additional risk alleles in patients with psoriasis and psoriatic arthritis. These alleles include interleukin-12A (IL12A), interleukin-12B (IL12B), IL23R, and genes that regulate NF-κB (296, 297). Obesity, severe psoriasis, other localised subtypes of psoriasis, trauma or deep lesions at the trauma site are all environmental risk factors that can lead to development of psoriatic arthritis (298, 299).

1.14.2 T cells in PsA and key cytokines

T cells are important players in psoriatic arthritis. The presence of association with HLA class I alleles as well as oligoclonal CD8+ T cell expansion support the hypothesis of a central role for CD8+ T cells in the pathogenesis of PsA. IL-17+CD8+ T cells and IL-17A/IL-22 producing cells known as type 3 innate lymphocytes are increased in psoriatic synovial fluid as compared with rheumatoid synovial fluid (15, 300, 301). Recent studies have shown that IL-23, IL-17 and TNFα are likely to be very important in the pathogenesis of PsA (15, 301, 302). IFN-α is also important in modulating inflammation. This cytokine was shown to activate dermal DCs, which triggered the differentiation of Th1 and Th17 cells in the lymph nodes. Th1 and Th17 cells were shown to migrate back to the dermis and to induce potent inflammatory responses (303).

1.15 Similarities and differences between RA and PsA

RA and PsA are two chronic inflammatory conditions characterised by both similarities and differences. Both diseases show signs of tender and soft tissue swelling of the joints but following a more accurate analysis the patterns of joint inflammation and damage in PsA are distinct from RA, and more closely match the clinical changes found in seronegative spondyloarthropathies. Contrary to RA, PsA joint pathology can include the axial skeleton, and specifically the sacroiliac joints and the lumbosacral spine, once again, matching what it is observed in ankylosing spondylitis (304). PsA and RA have different genetic associations with the major histocompatibility complex region that encodes HLA. RA is associated with HLA class II, whereas PsA is associated with HLA class I indicating a difference in genetic susceptibility and pathogenesis between the two diseases and suggesting that CD8+ T

cells, rather than CD4+ T cells, might have a central role in PsA (305-307). Furthermore, several genes associated with ankylosing spondylitis and inflammatory bowel disease such as the IL-23 receptor, CARD9 and ERAP1 have been also identified in patients with PsA (308). Direct comparison of synovial tissue biopsies from RA and PsA patients indicate that, as compared to RA, PsA display a less marked cellular infiltrate and a less extended hyperplasia of the lining layers of cells. Conversely, higher vascularisation can be found in PsA as compared to RA (309). In line with these findings increased expression of angiogenic growth factors including the vascular endothelial growth factor (VEGF), the angiopoietin 2 (Ang2), and the basic fibroblast growth factor (BFGF) are found in the PsA synovium as compared to RA (310, 311). As seen for RA, T cells have an important role in promoting inflammation in PsA as demonstrated by beneficial effects of therapies such as cyclosporine and anti-CTLA-4 (abatacept) in the treatment of PsA (312, 313). Specific subsets of T cells are likely responsible for promoting inflammation and disease activity as shown by work from Menon *et al.* which identified an enrichment of IL-17+CD8+ T cells in the synovial fluid of PsA patients but not in RA (15). Finally, although PsA is not associated with RA-specific circulating autoantibodies (i.e. RF and ACPA), it is reported that B-cell aggregates can be found both in PsA and RA synovial biopsies although it is not clear what is the role of B cells in PsA (314, 315). Once again, both similarities and differences can be found in the therapeutic responses of RA and PsA. Disease modifying anti-rheumatic drug (DMARD) methotrexate (MTX) and biologics such as tumour necrosis factor inhibitors (TNFi) appear to be effective in the management of both RA and PsA (316, 317). The fusion protein against CTLA-4 (abatacept) which acts by binding to CD80 and CD86 on

APCs, blocking the engagement of CD28 on T cells and preventing T cell activation, has also shown similar activity in both PsA and RA (48, 313, 318).

Conversely, different results have emerged from important clinical trials targeting the IL-17 and IL-12/IL-23 pathways. The importance of IL-17 and IL-12/IL-23 in the pathophysiology of PsA has led to the development of specific monoclonal antibodies targeting these cytokines. The fully humanised anti-IL-12/IL-23 antibody, ustekinumab, has been studied in two large phase III randomised clinical trials and is now approved for the treatment of psoriasis and psoriatic arthritis (319, 320). Similarly a monoclonal antibody toward IL-17A (sekukimumab) is now approved for the treatment of PsA (321) but not for RA after it failed to demonstrate major benefits in a phase III study in comparison with anti-CTLA-4 abatacept (322).

1.16 Thesis rationale and thesis aims.

Literature presented in this chapter indicates that PD-1 is an important regulator of T cell functions and that blockade or lack of this inhibitory receptor is directly associated with autoimmune conditions both in mouse and human. Several lines of evidence indicate that the frequencies of PD-1+CD4+ T cells are increased in RA synovial fluid (SF) compared to RA and healthy peripheral blood (PB) (216, 323, 324) while only limited data exists regarding the ability of SF-derived cells to be regulated via PD-1. An indication of a defective PD-1 pathway in the context of inflammatory arthritis comes from a study performed in RA showing that synovial fluid (SF) CD4+ T cells display reduced PD-1-mediated inhibition compared to RA PB cells (216). This suggests that under conditions of chronic inflammation the PD-1 pathway might be negatively modulated. In PsA, it is unclear whether PD-1+CD4+ T cells are increased at the site of inflammation and whether PD-1 is able to regulate their proliferation and cytokine production. Hence, further investigation is required.

Therefore, the first main aim of this thesis (chapter 3) was to extend data from previous investigations by examining PD-1 and PD-L1 expression in different cell subsets from both patients with RA or PsA. A minor aim was to assess the presence of any differences between these two arthrides.

The second major aim (chapter 4) was to explore whether PD-1 ligation in healthy PB and in RA- and PsA-derived PB and SF CD4+ T cells is able to modulate T cell proliferation and cytokine production. Further experiments were also designed to investigate whether PD-1-mediated regulation in healthy CD4+ T cells is affected by proinflammatory cytokines typically associated with RA and PsA.

The third and final aim (chapter 5) was to identify a mechanism by which proinflammatory cytokines modulate PD-1 ligation *in vitro* and to examine whether the above findings had a clinical relevance in RA and PsA settings. This included i) analysis of the presence of a soluble form of PD-1 (sPD-1) in both the serum and SF of RA and PsA patients, ii) preliminary analysis aimed at identifying differences between patient groups undergoing therapy with DMARDs vs. Biologics and iii) *in vitro* experiments to evaluate whether a soluble PD-1fc chimera, used to mimic naturally expressed sPD-1, modulates PD-1 mediated suppression of proliferation.

2 Materials and Methods

2.1 Human samples

Heparinised peripheral blood (PB) and matched synovial fluid (SF) samples (where available) were obtained from patients with osteoarthritis (OA), rheumatoid arthritis (RA) and psoriatic arthritis (PsA) recruited from the rheumatology outpatient clinic at Guy's and St. Thomas' Hospital NHS Trust (London, UK). Healthy control (HC) subjects were recruited from among local student and staff volunteers. Written informed consent was received from all participants. Ethics approval was given by the Bromley Research Ethics Committee (approval no. 06/Q0705/20). For OA samples, ethics approval was given by the Guy's Research Ethics Committee (01/05/01). All samples were collected in compliance with the Declaration of Helsinki. A small amount of peripheral blood (PB) was drawn into a BD Vacutainer® tube (Becton Dickinson, Franklin lakes, NJ, USA) containing no anticoagulant. Blood was incubated in an upright position at 4°C for 30-45 min to allow clotting and a small aliquot of serum (1-2 ml) was removed. The serum aliquot was centrifuged twice at 1200 rpm for 10 minutes and cell-free serum was stored at -80°C for analysis of cytokine and soluble PD-1 (sPD-1) content. Information on clinical and demographic parameters, where available, is provided in table 2.1.

	RA PB	RA SF	PsA PB	PsA SF
Sex (Female/Male)	29/5	23/2	11/17	11/17
Age in years (mean±SD)	54.5 (±16.5) (n=34)	58.7 (±15.5) (n=25)	39.8 (±12.4) (n=28)	39.8 (±12.4) (n=28)
DAS28 score (mean±SD)	5.0 (±1.82) (n=25)	5.0 (±1.82) (n=25)	4.5 (±1.23) (n=24)	4.5 (±1.23) (n=24)
Treatment (None/DMARD/ Biologic)	5/21/8	5/12/8	7/12/9	7/12/9
Rheumatoid Factor (+/-)	19/6	19/6	0/28	0/28

Table 2.1 Demographic and clinical parameters of the patients included in the study. Some samples were used for flow cytometry or functional assays only, whilst other samples were only used for cytokine detection in serum and SF. Clinical and demographic data are provided, where available. Abbreviations used: RA, rheumatoid arthritis; PsA, psoriatic arthritis; PB, peripheral blood; SF, synovial fluid; DAS28, disease activity score of 28 joints; DMARDs, disease-modifying anti-rheumatic drugs. DAS28 > 5.1 indicates highly active disease, 3.2 – 5.1 moderate activity, 2.6 - 3.2 low activity, <2.6 remission.

2.2 Cell Isolation

2.2.1 Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood mononuclear cells (PBMC) from healthy volunteers, osteoarthritis (OA), rheumatoid arthritis (RA) and psoriatic arthritis (PsA) patients were isolated using Lymphoprep™ (Axis-Schield, Oslo, Norway) by density-gradient centrifugation. Blood was diluted 1:1 in sterile phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) solution and layered on 15 ml of Lymphoprep™ reagent in 50ml falcon centrifuge tubes (Corning Inc. New York, USA). Tubes were centrifuged at 1600 rpm (515 x*g*) for 20 mins at room temperature using an Eppendorf

5810R centrifuge (Eppendorf, Hamburg, Germany). After centrifugation, the cloudy white layer of PBMC was collected using a sterile Pasteur pipette. The PBMC layer was transferred in a new sterile falcon centrifuge tube, washed twice at 1200 rpm (515 x*g*) for 10 minutes in PBS. Supernatants were removed and cell pellets resuspended in cold MACS buffer (sterile PBS containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 2 mM EDTA (Life Technologies, Paisley, UK)). Live cells were counted using trypan blue (Sigma-Aldrich) exclusion and adjusted to a concentration of 10 x 10^6/ml before proceeding to magnetic-activated cell sorting (MACS) isolation. Extra aliquots of PBMC were centrifuged and the pellets were resuspended in freezing solution, made up of fetal calf serum (FCS) and 10% Dimethyl sulfoxide (DMSO), at final concentrations of 20x10^6/ml. Cells were transferred to a -80 °C freezer for a minimum of 24 hours. The cryovials were then stored into liquid nitrogen tanks.

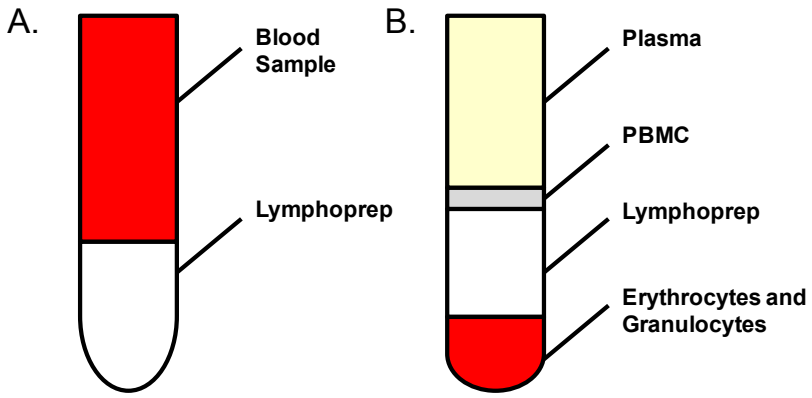

Figure 2.1 PBMC isolation via density gradient centrifugation.
Before centrifugation (A) and after centrifugation (B).

2.2.2 Synovial fluid mononuclear cell (SFMC) isolation

Synovial fluid was removed by needle aspiration and collected in 50 ml sterile tubes. A small aliquot of fluid (1-2 ml) was centrifuged at 1200 rpm for 10 minutes and cell-free fluid was collected and stored at -80°C for cytokine and soluble PD-1

(sPD-1) content analysis. The cell pellet from the small aliquot was resuspended in sterile PBS and added to the main synovial fluid sample before processing. Synovial fluid was diluted 1:2 in sterile PBS solution. In some cases of excessively viscous samples, a higher dilution (up to 1:5) was used. SFMC were isolated by density-gradient centrifugation as previously described in section 2.1.1 and counted using trypan blue. Extra aliquots of SFMC were cryopreserved as described in section 2.2.1.

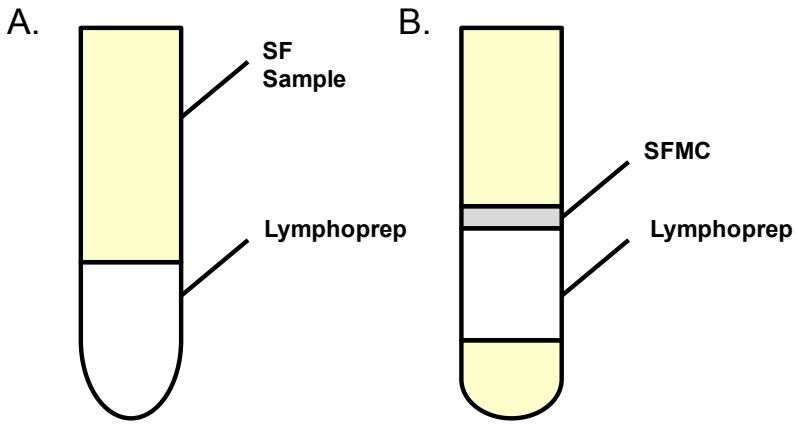

Figure 2.2 SFMC isolation via density gradient centrifugation.
Before centrifugation (A) and after centrifugation (B).

2.3 Isolation of different cell subsets

2.3.1 CD4+ T cell isolation

CD4+ T cells were negatively isolated by magnetic bead separation using the CD4+ T cell isolation kit (130-096-533) from Miltenyi Biotec (Bergisch Gladbach, Germany). PBMC or SFMC were isolated from PB and SF, respectively, as described in sections 2.2.1 and 2.2.2. Cells were then counted and incubated with a cocktail of biotin-conjugated monoclonal antibodies (mAb) against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A) for 5 minutes at 4°C. Subsequently, a MicroBead cocktail which binds non-target cells was added and

cells were further incubated at 4°C for additional 10 minutes. During the second incubation, a LS column (Miltenyi Biotech, Bergisch Gladbach, Germany) was placed in a QuadroMACS™ magnet (Miltenyi Biotech) and pre-wet with 3ml of MACS buffer composed by 0.5% BSA (Sigma-Aldrich), 2mM EDTA (Life Technologies, Carlsbad, USA) and PBS (Sigma-Aldrich). After the second incubation, the cell suspension was added directly to the LS column and the flow-through containing the unlabeled CD4+ T cells was collected. To wash the column, 3 ml of MACS buffer solution were applied and combined with the first flow-through. The purified CD4+ T cells eluted from the column were counted using trypan blue and stained for purity. CD4+ T cells purity was determined by flow cytometry (purity range 95-99%). In some cases HC CD4+ T cells were centrifuged and the pellets were resuspended in freezing solution, made up of Fetal Bovine Serum (FBS) and 10% Dimethyl sulfoxide (DMSO), at final concentrations of $20x10^6$/ml. Cells were transferred to a -80 C freezer for a minimum of 24hours. The cryovials were then stored into liquid nitrogen tanks.

2.3.2 CD4+CD25+ T regulatory (Treg) cell isolation

In some experiments, after CD4+ T cell isolation, a further step using the Dynabeads® Regulatory CD4+CD25+ T Cell Kit (11363D; Life Technologies, Carlsbad, USA) was performed in order to isolate CD4+ Treg cells. For Treg cells isolation 1.5×10^7 CD4+ T cells (volumes were scaled up accordingly to manufacturer's instructions) were incubated with pre-washed and resuspended Dynabeads® CD25 reagent for 25 min at 4°C with rolling and tilting. After this incubation, cells were put in a DynaMag™ magnet (Life Technologies) for a minimum of 2 minutes and the supernatant containing the CD4+CD25- cells was

carefully removed. The bead-cell complexes formed by the CD25 beads and the CD4+CD25+ Treg cells were then washed twice in MACS buffer and resuspended in 500 µl of RPMI (RPMI-1640; Gibco) with 1% FBS. Following this step, 80 µl of DETACHaBEAD® reagent was added and cells were incubated for 45 minutes at room temperature with tilting and rotation. Cells were then put again in the magnet for a minimum of 2 minutes and the supernatant containing the CD4+CD25+ Treg cells was carefully removed. Two final washes were then performed and supernatants were combined. The purified CD4+CD25+ T cells were then counted using trypan blue and stained for purity. Treg purity was determined by flow cytometry (purity >90%).

2.3.3 CD14+ cell isolation

In some experiments isolation of CD14+ cells was performed. CD14+ monocyte isolation was performed from HC PBMC purified as described in sections 2.1.1. Monocytes were positively isolated using a CD14 Microbeads kit (130-050-201; Miltenyi Biotec). HC PBMC were counted, resuspended in MACS buffer at the suggested volume and incubated with CD14+ Microbeads for 15 minutes at 4°C. Cells were then centrifuged at 300xg for 10 minutes and resuspended in MACS buffer. During this 15 minutes incubation, a LS column was placed in a QuadroMACS™ magnet (Miltenyi Biotech) and rinsed with 3ml of MACS buffer. Cell suspension was then applied to the column followed by 3 washes of 3 ml each. The column was removed from the magnet and placed in a collection tube. 5 ml of MACS buffer were applied to the column and the magnetically labelled CD14+ cells were flushed out using the provided plunger. Cells were then counted and stained for purity by flow cytometry (purity range 96-98%).

2.4 Cell Culture

All cells were cultured in Roswell Park Memorial Institute (RPMI) (RPMI-1640; Gibco) medium supplemented with 1% penicillin/streptomycin, 1% L-glutamine (Gibco) and 10% heat-inactivated fetal calf serum (Gibco) and maintained in an incubator at 37°C and 5% CO_2 atmosphere. Cells were plated at different concentrations ranging from $4x10^4$/ml to $1x10^6$/ml depending on the assay. Cells were typically cultured for 3 to 5 days depending on the chosen assay. In CD4+ T cell-only cultures cells were stimulated with plate-bound anti-CD3 mAb (OKT3, Janssen-Cilag Ltd, Wycombe, UK) (1.5 μg/ml) while in CD4+ T cell/CD14+ monocyte co-cultures, soluble anti-CD3 (OKT3, Janssen-Cilag Ltd, Wycombe, UK) (100 ng/ml) was added. Treg:Teff co-cultures cells were stimulated with either soluble anti-CD3 mAb (100 ng/ml) or with anti-CD3/anti-CD28 beads (Life Technologies) at a ratio of 1 bead for every 5 Teff cells. Where indicated, neutralising antibodies, recombinant cytokines or other reagents were added at the start of the culture period.

2.4.1 Cell culture reagents

The following reagents were used for cell culture according to the experiment.

Reagent	**Supplier**	**Amount used**
Lymphoprep™	Axis-Schield	15 ml / 50 ml
RPMI-1640	Gibco	As required
Penicillin/Streptomycin	Gibco	1%
L-glutamine	Gibco	1%
Phosphate buffer saline	Sigma-Aldrich	As required
BSA	Sigma-Aldrich	As required
Fetal Calf Serum	Gibco	10%

DMSO	Sigma-Aldrich	As required
Anti-CD3 mAb	Janssen-Cilag	As indicated
Anti-CD3/CD28 beads	Gibco	As indicated
[^{3}H]-thymidine	GE Healthcare	0.25 μCi/well
CellTrace™ Violet	Life Technologies	2μM

Table 2.2 List of culture reagents used for cell culture.

2.4.2 Cytokines

The following cytokines were added to specific cell cultures:

Cytokine	**Supplier**	**Concentration used**
TNFα	R&D Systems	10ng/ml
IL-6	R&D Systems	10ng/ml
IL-1β	R&D Systems	10ng/ml

Table 2.3 List of cytokines used during cell culture.

2.4.3 Antibodies

The following mAb antibodies were added to specific cell cultures:

Antibody	Effect	Supplier	Clone	Used at:
Anti-CD3	Stimulation	Janssen-Cilag	OKT3	100 ng/ml
Anti-CD3	Stimulation	Janssen-Cilag	OKT3	1.5 µg/ml
Anti-TNFα	Neutralisation	AbbVie	Adalimumab	1 µg/ml
Anti-IL-6R	Neutralisation	Roche	Tocilizumab	1 µg/ml
Anti-IL-1β	Neutralisation	R&D Systems	8516	1 µg/ml
Isotype	Null	R&D Systems	11711	1 µg/ml

Table 2.4 List of antibodies used in cell culture.

2.4.4 Chimera proteins

The following carrier free chimera proteins were used to coat 96 well plates or added in soluble form at the start of the cell culture. To control for the addition of the proteins the relevant control was used as listed.

Protein	Supplier	Concentration used
PD-L1fc	R&D Systems	Range: 0, 0.1, 1, 2 and 5 µg/ml
PD-1fc	R&D Systems	Range: 0, 0.25, 0.5, 1 and 2 µg/ml
IgG1fc	R&D Systems	Range: 0, 0.1, 0.25, 0.5, 1, 2 and 5 µg/ml

Table 2.5 List of chimera proteins used during cell culture.

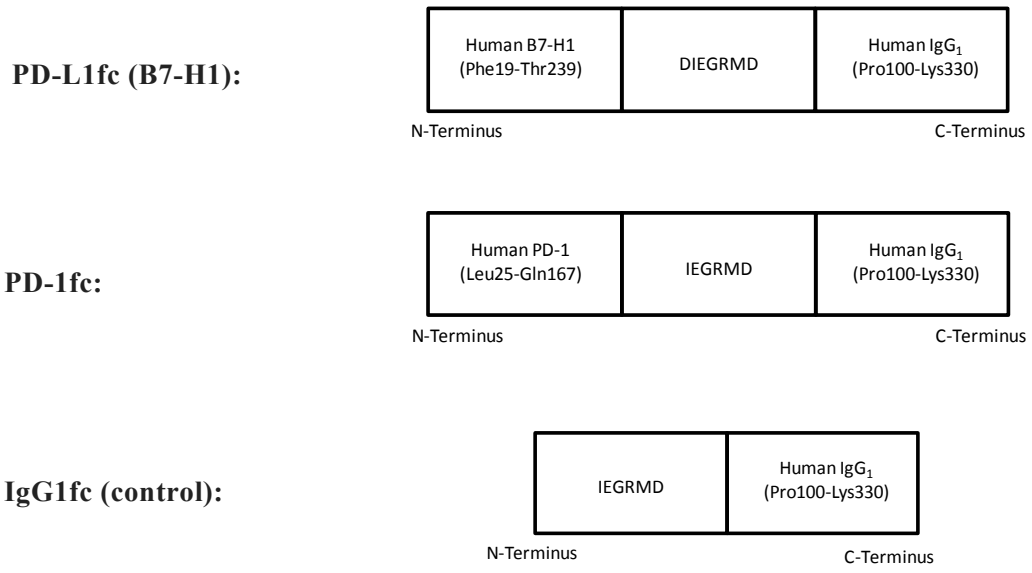

Figure 2.3 Schematic representation of the PD-L1fc and PD-1fc chimera and IgG1fc control.
The PD-L1fc chimera is composed by a human IgG1, a link peptide (DIEGRMD) and the extracellular portion of the PD-L1 ligand. The PD-1fc chimera is composed by a human IgG1, a link peptide (IEGRMD) and the extracellular portion of the PD-1 receptor. The IgG1fc control is composed by a human IgG1 and a link peptide (IEGRMD).

2.5 PD-1-mediated T cell suppression assay

To assess T cell suppression of proliferation in CD4+ T cell-only cultures, 96-well flat-bottom plates (Costar, Corning Inc., Corning, NY, USA) were coated with anti-CD3 mAb (1.5 μg/ml) and either PD-L1fc or IgG1fc (R&D Systems, Minneapolis, MN, USA) (range from 0 to 5 μg/ml according to the experiment) for 4 hrs at 37°C and 5% CO_2 in 100 μl final volume of sterile PBS. Plates were washed twice with sterile PBS before cells were added for culture. In T cell-only assays, CD4+ T cells were negatively isolated from cryopreserved HC PBMC, RA and PsA PBMC and RA and PsA SFMC as described in section 2.3.1 and cultured at a concentration of $1x10^5$ cells per well in a final volume of 200 μl of culture medium. At day 4, cells were pulsed with [^{3}H]-thymidine (0.25 μCi/well) (GE Healthcare, Chicago, IL, USA) and T cell proliferation was assessed after 18 hrs (on day 5) using a Topcount scintillation counter (Perkin Elmer, Waltham, MA, USA). Cells were harvested in a glass fiber filter (Perkin Elmer) and proliferation was determined as counts per minutes (cpm), a measure of the detection rate of ionization events per minute. In some cultures, human recombinant (hr) TNFα, hrIL-6 or hrIL-1β (all at 10 ng/ml, R&D Systems, Minneapolis, MN, USA) were added from the start of the culture in the absence or presence of anti-TNFα drug adalimumab (AbbVie, Lake Bluff, IL, USA), anti-IL-6R drug tocilizumab (Roche, Basel, Switzerland) and anti-IL-1β (R&D Systems) (all at 1 μg/ml).

Clinical grade therapeutics were purchased via Guy's Hospital Pharmacy and frozen in aliquots at -20°C. In some experiments, to assess T cell proliferation in presence of a soluble form of the PD-1 receptor, HC CD4+ T cells were cultured in PD-L1fc (0, 0.1 and 1 μg/ml)-coated plates in the presence of soluble PD-1fc chimera

or the appropriate control (0.5 and 1ug/ml, R&D Systems, Minneapolis, MN, USA). Proliferation was expressed as counts per minute (cpm) and as suppression of T cell proliferation (%) according to the following formula:

[(medium only condition − PD-L1fc condition) / medium only condition] × 100

The schematic representation of the described experiments is shown in Figures 2.4A and 2.4B.

2.6 T cell:Monocyte co-culture assay

This co-culture set up was used to test the effect of soluble PD-1fc chimera (sPD-1) in a T cell/APC co-culture system. Freshly isolated HC CD4+ T cells and autologous CD14+ monocytes were co-cultured in 96-well flat bottom plates (Costar) at 1:1 and 1:0.5 ratio (total cells per well $1x10^5$) in 200 µl final volume of culture medium containing 100 ng/ml soluble anti-CD3 mAb and sPD-1fc or IgG1fc (0, 0.25, 0.5 and 1 µg/ml). In all assays, at day 4, cells were pulsed with [^{3}H]-thymidine (0.25 µCi/well) and T cell proliferation was assessed after 18 hrs (on day 5) using a Topcount scintillation counter. Proliferation was expressed as counts per minutes (cpm) and as suppression of T cell proliferation (%) according to the formula reported in section 2.5. The schematic representation of the described experiments is shown in Figure 2.5A.

2.7 Treg:Teff cell suppression assay

Suppression assays were carried out using freshly isolated whole PBMCs as T effector (Teff) cells and freshly isolated CD4+CD25+ cells as regulatory T cells (Treg). Up to $10x10^6$ Teff cells were stained with 2 µM of CellTrace™ Violet (CTV) dye (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's

protocol. Subsequently, cells ($4x10^4$/well) were cultured in 96 well U-bottom plates in 200 μl final volume of culture medium containing 100 ng/ml soluble anti-CD3 mAb or anti-CD3/anti-CD28 beads (1 bead every 5 cells; 8000 beads/well) in the absence or presence of autologous CD4+CD25+ Treg cells ($2x10^4$/well and $1x10^4$/well equivalent to 1:0.5 and 1:0.25 Teff/Treg ratio). Teff cells were also cultured alone at $6x10^4$/well (1.5:0 Teff/Treg ratio) to control for cell overcrowding in the wells. In some experiments, the sPD-1fc chimera or the sIgG1fc control were added from the start of the culture (1 μg/ml and 2 μg/ml, R&D Systems). Cells were cultured for 48 hours and the proliferation percentage of CD4+ and CD8+ T cells within PBMC Teff population was determined by flow cytometry as CTV dye dilution. The schematic representation of the described experiments is shown in Figure 2.5B.

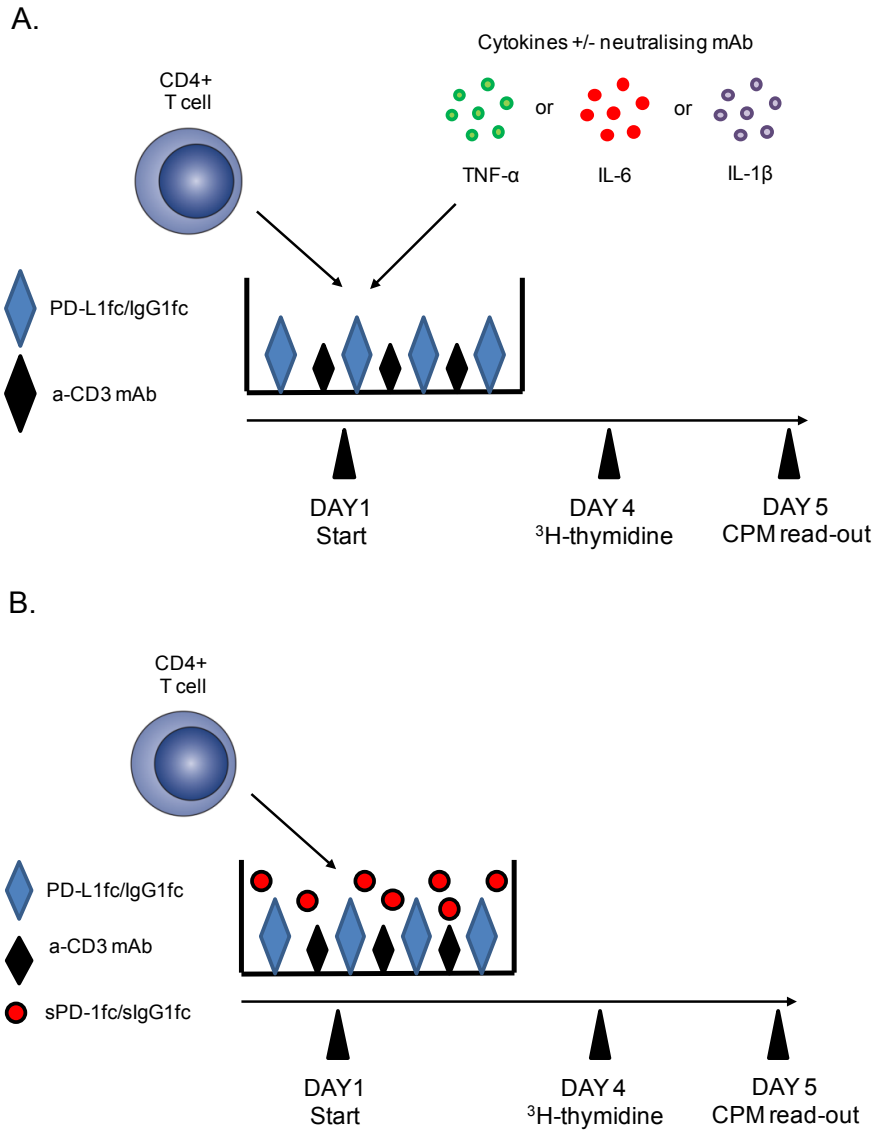

Figure 2.4 Schematic representation of the CD4+ T cell suppression assay in presence of the PD-L1fc chimera.
CD4+ T cells were isolated from HC PBMC, RA and PsA PBMC and SFMC and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc/IgG1fc (0, 0.1, 1, 2 and 5 µg/ml). Proliferation was assessed on day 5 by [^{3}H]-thymidine incorporation. Cells were cultures with ± cytokines and ± neutralizing mAb (A) or ± sPD-1fc/IgG1fc (B) from the start of the culture.

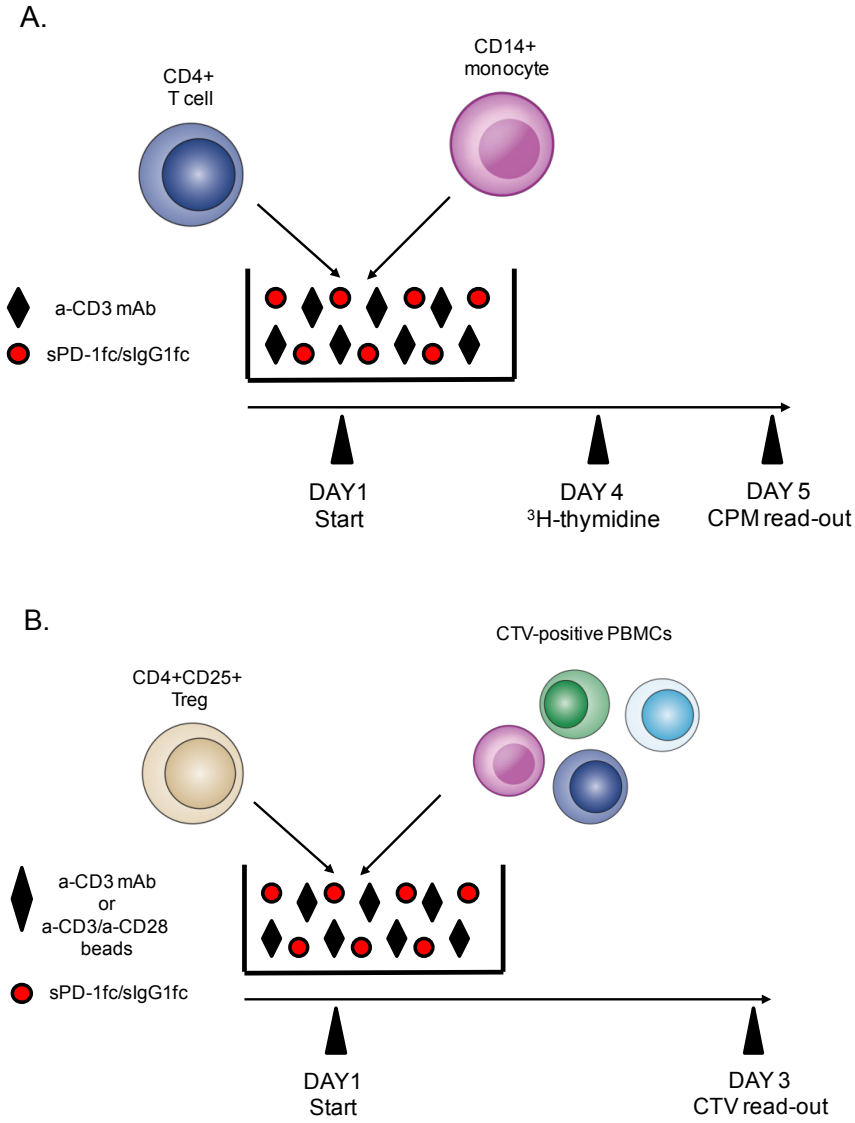

Figure 2.5 Schematic representation of the T cell:Monocyte co-culture and Treg:Teff cell suppression assay in presence of the sPD-1fc chimera.
(A) CD4+ T cells and autologous CD14+ monocytes were isolated from HC PBMC and cultured for 5 days at 1:1 and 1:0.5 ratios in the presence of soluble anti-CD3 mAb (100 ng/ml) and soluble PD-1fc/IgG1fc control (0, 0.25, 0.5 and 1 µg/ml). (B) Whole PBMC Teff cells from healthy controls were labelled with cell trace violet (CTV) and cultured in the presence of anti-CD3 monoclonal antibody (100 ng/ml) or anti-CD3/CD28 beads (1 bead every 5 cells), ± autologous PB CD4+CD25+ Treg cells and ± PD-1fc (1 and 2 µg/ml) or IgG1fc (2 µg/ml).

2.8 Cytokine analysis

2.8.1 Introduction

ELISA assay: The Enzyme-linked immunosorbent assay (ELISA) is a method used to detect soluble proteins like cytokines, chemokines and growth factors in a biological sample of interest. To perform an ELISA assay, a capture antibody specific for an analyte of interest is used to coat a 96 well ELISA plate. The plate is then blocked for a minimum of 1 hour with protein (i.e. 1% Bovine Serum Albumin in PBS) to prevent unspecific interaction. The sample of interest is then added and incubated for about 2 hours. The analyte of interest present in the sample will bind the capture antibody. Then, a secondary biotinylated detection antibody is added to the plate for a time, which can range from 1 to 2 hour according to the assay. The secondary antibody will bind the analyte of interest at a different epitope then the one recognized by the capture antibody. Finally, streptavidin-HRP followed by a chromogenic substrate is added to allow colour visualization. The optical density of each well is then determined using a microplate spectrophotometer reader set to 450 nm.

Luminex assay: The Luminex assay is based on three core elements: Antibody-coated microspheres which are dyed to emit fluorescence and which have distinctive colour codes or spectral addresses to allow discrimination and simultaneous detection of multiple analytes. A specific flow cytometer to measure the specific molecules bound on the beads surface and a digital signal processor to analyse the fluorescence data. In this specific assay, beads are coupled with specific capture antibodies directed towards different biomarkers of interest. The beads react with the samples containing the specific biomarker forming a bead-biomarker bound. Residual un-bound beads are then removed with washing steps and a secondary

biotinylated detection antibody is added to create a complex as previously described for the ELISA assay. Finally, Streptavidine-Phycoerytrin (SA-PE) conjugate is added to provide the fluorescent signal.

2.8.2 *Ex vivo* analysis of cytokine and soluble PD-1 levels

Cell free serum and synovial fluid were collected as described in sections 2.1 and 2.1.2 and kept at -80°C until the specific analysis was performed. Serum samples from HC donors and serum and paired cell-free synovial fluid samples from patients with OA, RA or PsA were thawed at room temperature before each analysis. For cytokine detection using Luminex technology, samples were diluted 1:2 in the dilution buffer provided by the analysis kit. The cytokine levels in the samples were tested using the Bio-Plex Pro™ (Bio-Rad, Hercules, CA, USA) kit and analysed on the Luminex FlexMap 3D platform (Luminex Corporation, Austin, TX, USA). The assay and the data analysis were performed according to the manufacturer's protocol. For soluble PD-1 (sPD-1) detection using the ELISA technique, serum and SF samples were diluted 1:10 and 1:40 in PBS supplemented with 10% fetal calf serum (FCS) as recommended by the manufacturer's protocol. Samples were run at two different concentrations to ensure they fell in the linear part of the standard curve. A serial dilution starting at 10,000 pg/ml of the provided standard was performed in each assay to create the standard curve. Finally, the optical density of each well was determined using a microplate spectrophotometer reader (Wallac 1420 Viktor, Perkin Elmer) set to 450 nm.

2.8.3 Post-culture analysis of cytokine and soluble PD-1 levels

T cell culture supernatants were collected at day 5, spun to remove cellular debris, and stored at −80°C until analysis. IFN-γ levels from HC, RA and PsA CD4+ T cell cultures and IL-10 levels from HC CD4+ T cell cultures stimulated with TNFα and IL-6, were determined by ELISA using the ELISA MAX™ standard sets (Biolegend, San Diego, CA, USA). A serial dilution of the provided standard was performed in every assay and cell culture supernatants were diluted 1:10 and 1:20 before each assay. In some cases, HC CD4+ T and RA or PsA PBMC and paired SFMC culture supernatants were tested using the Bio-Plex Pro™ kit and analysed on the Luminex FlexMap 3D platform as previously described in section 2.8.2. HC CD4+ T cell culture supernatants were also tested (neat) for sPD-1 using the Human PD-1 DuoSet ELISA (R&D Systems) and analysed as described in section 2.8.2.

2.8.4 ELISA and LUMINEX kits

The following ELISA and Luminex kits were used to assess cytokine levels in Serum and SF of HC, RA and PsA donors and in HC CD4+ T cell cultures.

Target	Supplier	Assay
IFN-γ	Biolegend	ELISA
IL-10	Biolegend	ELISA
sPD-1	R&D	ELISA
TNFα	BioRad	Luminex
IL-6	BioRad	Luminex
IL-1β	BioRad	Luminex

Table 2.6 List of target molecules and respective assay performed for detection.

2.9 Flow Cytometry

2.9.1 Introduction

In all multicolour flow cytometry experiments, single-stained compensation controls were used to optimise fluorescence compensation. BD CompBeads (BD Biosciences) were stained with fluorescently conjugated antibodies, according to manufacturer's instructions. For the viability dye, a single-stained control was prepared using a mixture of live cells and cells that had been heat-killed at 65°C for ≥ 1 minute. Single-stained controls for cellular proliferation dye (CTV) were prepared by pooling unlabelled cells with dye-labelled, unstimulated cells.

2.9.2 Extracellular staining (*ex vivo*)

For *ex vivo* analysis of frequency and phenotype of each cell subset, PBMC or SFMC were extracellularly stained using antibodies conjugated to fluorochromes. Cells (approximately $5x10^5$ per stain) were washed in fluorescence-activated cell sorting (FACS) buffer composed by PBS plus 1% BSA (Sigma-Aldrich) and 0.1% sodium azide (Severn Biotech, Kidderminster, UK) and then stained with specific antibodies against CD3, CD8, CD4, CD14, CD25, CD127, CD274 (PD-1) and CD279 (PD-L1) (See Section 2.9.5). Isotype controls and fluorescence-minus-one (FMO) controls were also included in the staining. Cells were incubated at 4°C for 30 minutes to allow specific binding of the selected markers followed by two washes in FACS buffer at 1400 rpm for 8 minutes to remove unbound antibody. Cells were then fixed in a 2% paraformaldehyde (PFA) solution (Merck, Kenilworth, NJ, USA) for 15 min at 4°C, washed twice with FACS buffer and resuspended in 200 µl of FACS buffer for subsequent acquisition by flow cytometry.

2.9.3 Extracellular staining (post-culture)

PBMC responders from Treg/Teff co-cultures (see section 2.7) were stained extracellularly with a cocktail of antibodies to assess CTV dye dilution for CD4+ and CD8+ cells within the Teff population. Cells from each experimental condition (this included unstimulated Teff, stimulated Teff and stimulated Teff + Treg cells) were transferred from a 96 well plate into specific labelled FACS tubes. Cells were washed with FACS buffer at 1400 rpm for 8 minutes and stained with a cocktail of antibodies against CD4, CD8 and CD14. In some case a live/dead dye (eBioscience, San Diego, CA, USA) was included in the stain to gate out both dead cells and monocytes. Cells were incubated at 4°C for 30 minutes to allow specific binding of the selected markers followed by two washes in FACS buffer at 1400 rpm for 8 minutes each to remove the unbound antibodies. Finally cells were fixed in 2% PFA (Merck) and washed twice in FACS buffer for subsequent acquisition by flow cytometry. A representative staining plot of an extracellular staining post-culture is shown in Figure 2.6 while representative plots of extracellular staining ex-vivo are shown in chapter 3.

2.9.4 Acquisition and analysis

Cells were acquired (at least 10.000 total events) using a BD FACSCalibur or a BD FACSCanto II (Beckton Dickinson, Franklin lakes, New Jersey, USA) and analysed using the FlowJo software (version 7.6.5; Tree Star, Oregon, USA).

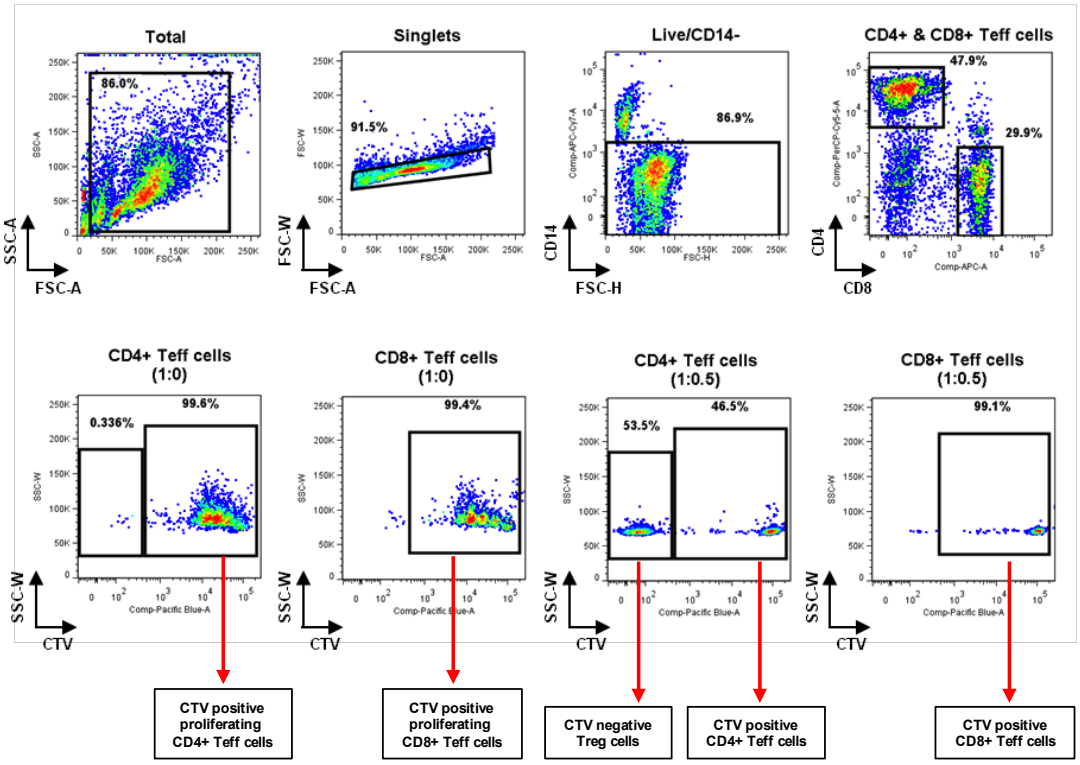

Figure 2.6 Schematic representations of one extracellular staining (post-culture) from a Treg:Teff suppression assay.
Stimulated CTV+ PBMC cultured in presence or absence of autologous Treg cells at Teff:Treg ratio of 1:0 and 1:0.5. Cells were gated as follows: Total cells, Singlets, CD14-/live cells, CD4+ or CD8+. CTV dilution for CD4+ and CD8+ cells is shown as a dot plot.

2.9.5 Flow cytometry antibodies

The following fluorochrome-tagged antibodies were used for extracellular staining.

mAb	Conjugate	Supplier	Isotype	Clone	per stain
CD3	PE/Cy7	Biolegend	mIgG1, κ	UCHT1	1 µl
CD4	PerCP/Cy5.5	Biolegend	mIgG1, κ	SK3	1 µl
CD8	APC	Biolegend	mIgG1, κ	SK1	1 µl
CD8	Pacific Blue	Biolegend	mIgG1, κ	RPA-T8	1 µl
CD14	Vio770	Miltenyi	mIgG2aκ	TÜK4	5 µl
CD25	APC	Biolegend	mIgG1, κ	BC-96	5 µl

CD127	PE	BD Pharmingen	mIgG1, κ	HIL-7R-M2	5 µl
CD127	FITC	Biolegend	mIgG1, κ	A019D5	1 µl
CD274 (PD-L1)	PE	BD Pharmingen	mIgG1, κ	MIH1	20 µl
CD279 (PD-1)	FITC	Biolegend	mIgG1, κ	EH12.2H7	5 µl
Isotype	PE	BD Pharmingen	mIgG1, κ	MOPC-21	20 µl
Isotype	FITC	BD Pharmingen	mIgG1, κ	MOPC-21	5 µl

Table 2.7 List of antibodies used for extracellular flow cytometry.

2.10 Quantitative polymerase chain reaction (qPCR)

2.10.1 RNA extraction and cDNA reverse-transcription

Total RNA was isolated with a ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's protocol. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA purity was assessed by analysing the ratio of absorbance at 260 nm and 280 nm and at 260nm and 230nm. A 260/280 ratio of ~2.0 is generally accepted as "pure" for RNA while values lower than that denote presence of protein, phenol or other contaminants. The 260/230 ratio is a secondary indication of purity and expected 260/230 values are commonly in the range of 2.0-2.2. RNA that met the purity criteria was then reverse-transcribed into cDNA. cDNA synthesis was performed using the high capacity cDNA reverse transcription kit (Applied Biosystem, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was stored at -20°C until the PCR assay was performed.

2.10.2 qPCR protocol and analysis

QPCR reactions were performed using the SensiMix™ SYBR No-ROX Kit (Bioline, London, UK) and with specific primers for human PD-1Δex3 and β-Actin (Integrated DNA Technologies IDT, Heverlee, Belgium). Reactions were performed on a Rotor-gene® Q real-time PCR cycler (Qiagen, Hilden, Germany) according to the manufacturer's protocol and data were analysed using the Rotor-Gene® Q Software version 2.1.1.49 (Qiagen). Thermocycler conditions included an initial holding at 95°C for 10 min, which was followed by a cycling program of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 50 cycles. The β-Actin gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample. All values were expressed as folds relative to the expression of β-Actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (CT). The amount of gene expression was then calculated as the difference (ΔCT) between the mean CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (β-Actin). Relative gene expression was expressed as $2^{-\Delta CT}$. To ensure a specific amplification of the PD-1Δex3 splice variant, the different primer sets were designed to anneal at the junction of the two exons spliced together (2 and 4) for PD-1Δex3. Sequence alignment was verified using the ApE software: http://biologylabs.utah.edu/jorgensen/wayned/ape/.

Target	Forward	Reverse
PD-1Δex3	5'-AGGGTGACAGGGACAATAGG-3'	5'-CCATAGTCCACAGAGAACAC-3'
β-Actin	5'-ATTGGCAATGAGCGGTTC-3'	5'-CGTGGATGCCACAGGACT-3'

Table 2.8 Primers sequences for PD-1Δex3 splice variant and β-Actin.

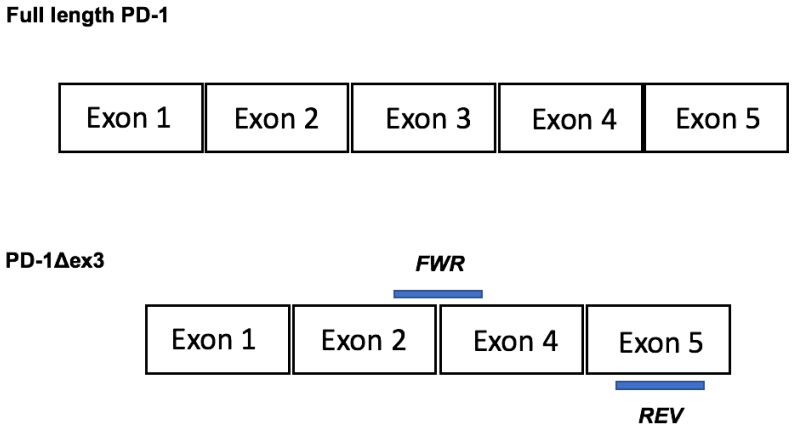

Figure 2.7 Schematic representations of primers, exon organisation and primers binding sites for PD-1Δex3.
Schematic representation of primers binding sites for PD-1Δex3 (FWR: Positions 495–504 and 661–670; REV: Positions 720–739).

The specificity of the selected primers for PD-1Δex3 was further tested as follows: 2 grams of agarose (Invitrogen, Life Technologies) were dissolved in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) to obtain a 2% agarose gel. The fluorescent nucleic acid dye GelRed™ (Thermo Fisher Scientific) was added to visualise the DNA. Quantitative PCR products with an appropriate DNA ladder for reference were run on the 2% agarose gel for 1 hour at room temperature and the gel was visualised using a Gel Doc™ EZ imager (Bio-Rad, Hercules, CA, USA). A representative gel image showing the primers specificity for PD-1Δex3 amplification can be found in chapter 5.

2.11 Genome-wide gene expression profiling

Gene expression profiling **data set #1**: CD25+CD127^{low} Treg cells and CD25−CD127+ Teff cells were sorted from the CD45RA+CD45RO− (naïve) or CD45RA−CD45RO+ (memory) CD4+ T cell compartments from the PB of healthy

controls and RA patients and a genome-wide expression profiling was carried out by Dr. Gina Walter (325) in the laboratory of Prof. Leonie Taams. A subset of the generated data were analysed by me and used for the purpose of this thesis to evaluate PD-1 and PD-L1 gene expression in CD4+CD45RO- (naïve) and CD4+CD45RO+ (memory) T cell compartments from the peripheral blood (PB) of healthy ad RA donors (Chapter 3; section 3.2.1; Figures 3.1 and 3.2). The demographics of the patients used for this analysis can be found here: (325).

Gene expression array profiling **data set #2**: PBMC and SFMC were collected from the blood and SF of 5 RA donors and Teff cells (CD25-CD127+) and Treg cells (CD25+CD127-) were sorted within the CD4+CD45RO+ population by Dr. Veerle Fleskens (unpublished data) in the laboratory of Prof. Leonie Taams. Genome-wide expression profiling was performed by Dr. Matt Arno in the KCL Genomics Centre using the Affymetrix HuGene 2_0st array. The freely available software from Affymetrix 'Expression Console' was used to perform the Quality control (QC) analysis. Qlucore Omics Explorer was used to perform the gene expression analysis. A subset of the generated data were analysed and graphed by me and used for the purpose of this thesis to evaluate PD-1 and PD-L1 gene expression in CD4+CD45RO+ (memory) T cell compartments from the peripheral blood (PB) and synovial fluid (SF) of RA patients. The demographics of the patients used for this analysis are shown in table 2.9.

Gene expression array profiling **data set #3**: CD14+ monocytes were sorted from the PB of healthy volunteers and from the PB ad SF of patients with RA and a third genome-wide gene expression profiling was performed by Dr. Megha Rajasekhar (250) in the laboratory of Prof. Leonie Taams. A subset of the generated data were analysed by me and used for the purpose of this thesis to evaluate PD-1 and

PD-L1 gene expression in HC PB, RA PB and RA SF-derived CD14+ monocytes. The demographics of the patients used for this analysis can be found here: (250).

	Age	Gender	DAS28 score	Treatment
RA_210	70	F	4.63	Embrel/Prednisone
RA_216	75	F	5.2	MTX, HCQ, SSZ
RA_308	71	M	6.05	Azathioprine
RA_322	71	F	3.5	ADA, SSZ
RA_330	65	M	6.9	MTX, Abatacept

Table 2.9 Demographic and clinical parameters of the patients included in the gene expression array #2.
Chapter 3, section 3.2.2, Figure 3.4). Abbreviations used: DAS28, disease activity score of 28 joints; Embrel, etanercept (anti-TNF); MTX, methotrexate; HCQ, Hydroxychloroquine; SSZ, Sulfasalazine; ADA, Adalimumab (Humira, anti-TNF); Abatacept (Orencia, CTLA-Ig). DAS28 > 5.1 indicates highly active disease, 3.2 – 5.1 moderate activity, 2.6 - 3.2 low activity, <2.6 remission.

2.12 Statistical analysis

Significance testing was performed with GraphPad Prism software (Version 7; GraphPad) using the appropriate statistical tests, as indicated in the figure legends. Comparative analysis between paired conditions (for example variations in percentage of a specific cell population between PB and SF of a same donor) was conducted using the Wilcoxon non-parametric t test. Comparisons between non-paired groups (for example patients treated with TNFi versus patients not treated with TNFi) were conducted using the Mann-Whitney non-parametric unpaired t test. In each analysis P values < 0.05 were considered to be statistically significant.

3 Results from a phenotypic and gene expression analysis indicate that PD-1 expression is increased at the site of inflammation in both RA and PsA

3.1 Introduction

T cell activation is a highly regulated process that involves the interplay between many different molecules. Activation of T lymphocytes generally requires two signals, the first signal is provided by the engagement of the T-cell receptor (TCR) with an MHC–peptide complex on an antigen-presenting cell (APC). The second signal is delivered upon engagement of a co-stimulatory receptor on T cells with a ligand on the APC. A key costimulatory signal is provided by the interaction between CD28 on T cells with ligands CD80 and CD86 expressed by antigen presenting cells (APCs) (326-330). It has been proposed that in patients affected by RA and PsA, costimulatory receptors and ligands such as CD86, CD28 and ICOS are overexpressed on T cells, macrophages and in the synovial tissue (331-335). These observations suggest that these molecules can contribute to persistent activation of T cells and to disease progression.

T cell activation is kept under control by CTLA-4 and PD-1, two important inhibitory receptors typically found on activated T cells (24, 36, 42, 43, 68, 336, 337). Over the past 10 years, the scientific community has shown an increased interest in understanding how chronic inflammation modulates the expression of PD-1 and its ligand PD-L1 in human inflammatory arthritis. In early studies performed on arthritis patients, it was found that PD-1+CD4+ T cells are increased in the synovial fluid (SF) of RA patients as compared to PB-derived cells from healthy controls (HC) and PB- and SF-derived cells from osteoarthritis (OA) controls (323, 324). RA SF-derived PD-

1+CD4+ T cells are for the most part memory cells (CD45RO+) and they can express the chemokine receptor CXCR3 (323). Increased percentages of PD-L1+ monocytes have also been described in the synovial fluid of certain groups of patients, but data are limited and in some cases contradictory. Yet, despite increased levels of PD-1 at the site of inflammation, it is unclear why the immune system fails to regulate synovial T cell activation. In RA, PD-1 and PD-L1 expression is still widely unexplored in cell types other than bulk CD4+ T cells. Furthermore, while this study was performed, the expression of PD-1 and PD-L1 in other arthrides such as psoriatic arthritis (PsA) had not been comprehensively explored.

Therefore, the aims of this chapter were:

1. To investigate PD-1 and PD-L1 gene expression in PB naïve and memory T effector (Teff) and T regulatory (Treg) cells from RA patients and healthy volunteers.
2. To investigate PD-1 and PD-L1 gene expression in paired PB and SF memory Teff and Treg cells from RA patients.
3. To investigate PD-1 and PD-L1 gene expression in PB and SF monocytes from RA patients and in PB monocytes from healthy volunteers.
4. To analyse, by flow cytometry, the expression levels of PD-1 and PD-L1 in PBMC and SFMC cell subsets from RA and PsA patients.

3.2 Results

3.2.1 Gene expression analysis for PD-1 and PD-L1 in PB CD45RA+ and CD45RO+ Treg cells and Teff cells from RA patients and healthy controls

The first aim of this chapter was to evaluate whether Teff and Treg cells from RA patients were different from HC cells at a molecular level regarding PD-1 and PD-L1 expression. For this purpose, data were collected and analysed from a previously generated gene expression profiling dataset (325).

A heat map, representing results from hierarchical cluster analysis, was generated to show the relative expression levels of PD-1 and PD-L1 in the different T cell subsets from the PB of healthy controls and RA patients (Figure 3.1). The heat map analysis revealed a very similar, almost identical, expression of *PDCD1* (PD-1) and *CD274* (PD-L1) between HC and RA T cell subsets while the highest expression of both receptor and ligand was observed specifically in memory (CD45RO+) Teff cells (Figure 3.1). Paired data analysis revealed that PD-1 expression was significantly higher in HC and RA memory (CD45RO+) Teff cells as compared to naïve cells (CD45RA+). PD-1 expression was also higher in HC and RA memory Treg cells as compared to naïve cells but the observed differences were not statistically significant (Figure 3.2A). Next, PD-L1 gene expression was analysed in the different subsets. PD-L1 was significantly increased in both memory Teff and memory Treg cells as compared to naïve cells (Figure 3.2B). No statistically significant differences for PD-1 or PD-L1 expression were found when comparing Teff or Treg cells (both naïve and memory) between HC and RA donors or when comparing PD-1 and PD-L1 expression between Teff and Treg cells (false discovery rate 5%, fold change in gene expression 1.5).

The *ex vivo* gene expression profiles for PD-1 and PD-L1 of Teff and Treg cells presented in this section indicate that no significant difference exists between the PB of RA patients and healthy controls. However, the higher expression levels of PD-1 and PD-L1 observed within the memory cell compartment of both HC and RA, as compared to naïve cells, might indicate that antigen-experienced T cells are likely to use the PD-1 signalling pathway to modulate their activation to a greater extent than cells that have not been exposed yet to antigens in the periphery.

Figure 3.1

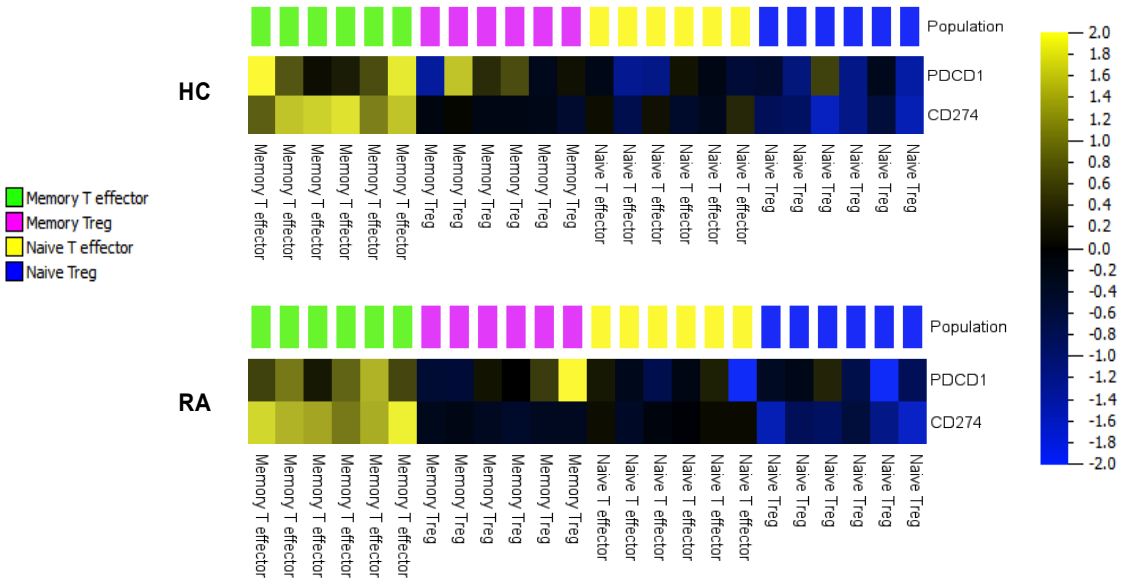

Figure 3.1 Gene expression profiling of naïve (CD45RA+) and memory (CD45RO+) Treg and Teff CD4+ T cell subsets from the PB of HC and patients with RA.
CD25+CD127^{low} Treg cells and CD25−CD127+ Teff cells were sorted from the CD45RA+CD45RO− (naïve) or CD45RA−CD45RO+ (memory) CD4+ T cell compartments from the PB of healthy controls and RA patients (n = 6) (325). The heatmap represents results from the hierarchical cluster analysis and shows the relative expression levels of *PDCD1* (PD-1) and *CD274* (PD-L1) genes in the different T cell subsets from the PB of healthy controls and RA patients. The demographics of the patients used for this analysis can be found here: (325).

Figure 3.2

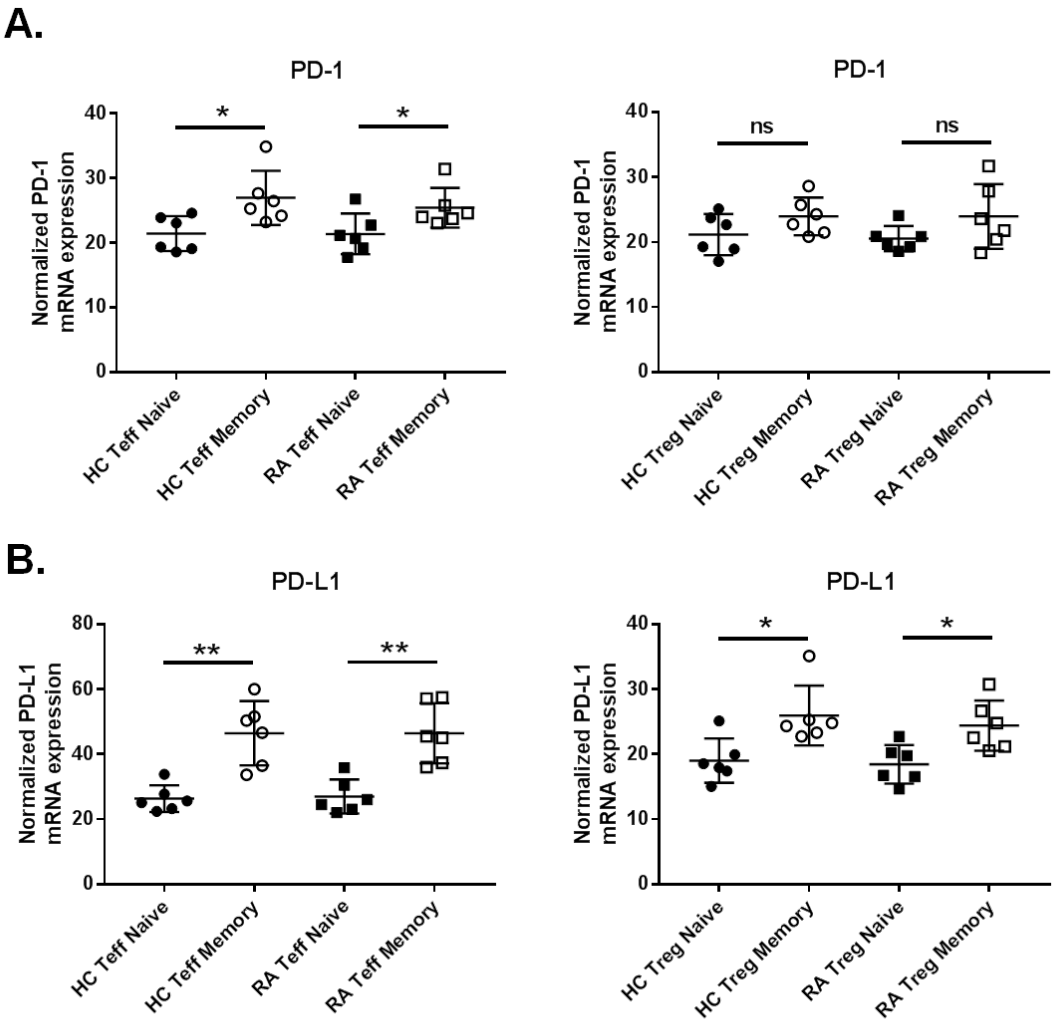

Figure 3.2 PD-1 and PD-L1 mRNA expression of naïve (CD45RA+) and memory (CD45RO+) Treg and Teff CD4+ T cell subsets from the PB of HC and patients with RA.

(A) Expression levels of PD-1 in HC and RA naïve and memory Teff cells and in naïve and memory Treg cells. (B) Expression levels of PD-L1 in HC and RA naïve and memory Teff cells and in naïve and memory Treg cells. HC and RA (n=6). RA n=7; PsA n=8) PB and SF cell populations. Data were analysed by Mann-Whitney test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3.2.2 Gene expression analysis for PD-1 and PD-L1 in PB and SF CD45RO+ Treg and Teff cells from RA patients

The second aim of this chapter was to evaluate the gene expression of *PDCD1* (PD-1) and *CD274* (PD-L1) in Teff and Treg cells from the PB and SF of RA patients. For this purpose, PD-1 and PD-L1 data were collected and analysed from a gene expression profiling dataset previously generated by Dr. Veerle Fleskens (unpublished data). In this specific array PBMC and SFMC were collected from the blood and SF of 5 RA donors. Teff cells (CD25-CD127+) and Treg cells (CD25+CD127-) were sorted within the CD4+CD45RO+ population. Figure 3.3 shows the gating strategy for the sorting of the different cell subsets. Data analysis showed that in 4 of 5 donors tested, PD-1 expression was increased in SF-derived Teff and Treg cells as compared to PB-derived cells (Figure 3.4A). Similarly, PD-L1 expression was also increased at the site of inflammation. PD-L1 expression was higher in 4 of 5 donors in SF-derived Teff cells and in 5 of 5 donors in SF-derived Treg cells as compared to PB-derived cells (Figure 3.4A). These data suggests that exposure to the highly proinflammatory environment found in the inflamed joint of RA can induce transcription of both the *PDCD1* (PD-1) and *CD274* (PD-L1) genes. No differences were found in the expression of PD-1 between Teff cells and Treg cells in the PB or SF (Figure 3.4B). On the contrary, PD-L1 expression was higher in Teff cells as compared to Treg cells in both the PB and the SF but only in the PB this increase reached statistical significance (Figure 3.4B). The *ex vivo* gene expression profiles for PD-1 and PD-L1 of Teff and Treg cells presented in this section show that RA SF-derived cells have higher expression of PD-1 and PD-L1 as compared to PB-derived cells. The data also show that PD-L1, but not PD-1, is increased in Teff compared to Treg cells suggesting a possible biological activity mediated by the PD-L1 ligand in Teff cells.

Figure 3.3

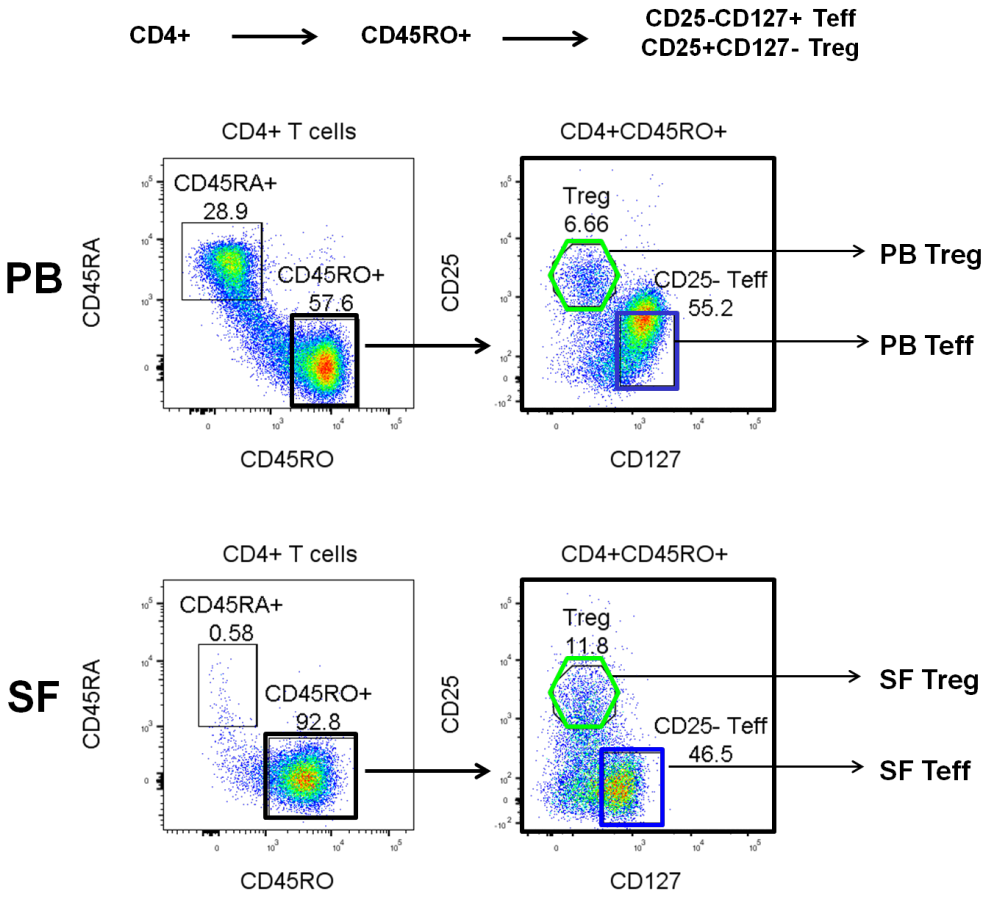

Figure 3.3 Gating strategy for the cell sorting of RA PB and SF CD25-CD127+ Teff and CD25+CD127- Treg cells for the generation of a gene expression profiling dataset.
Flow cytometry plots showing the gating strategy for the cell sorting. Cells were gated visually on total lymphocytes followed by CD4+ cells and CD45RO+ cells versus CD45RA+ cells. Next, Teff cells (CD25-CD127+) and Treg cells (CD25+CD127-) cells were gated within the CD4+CD45RO+ cell population and cell sorting was performed.

Figure 3.4

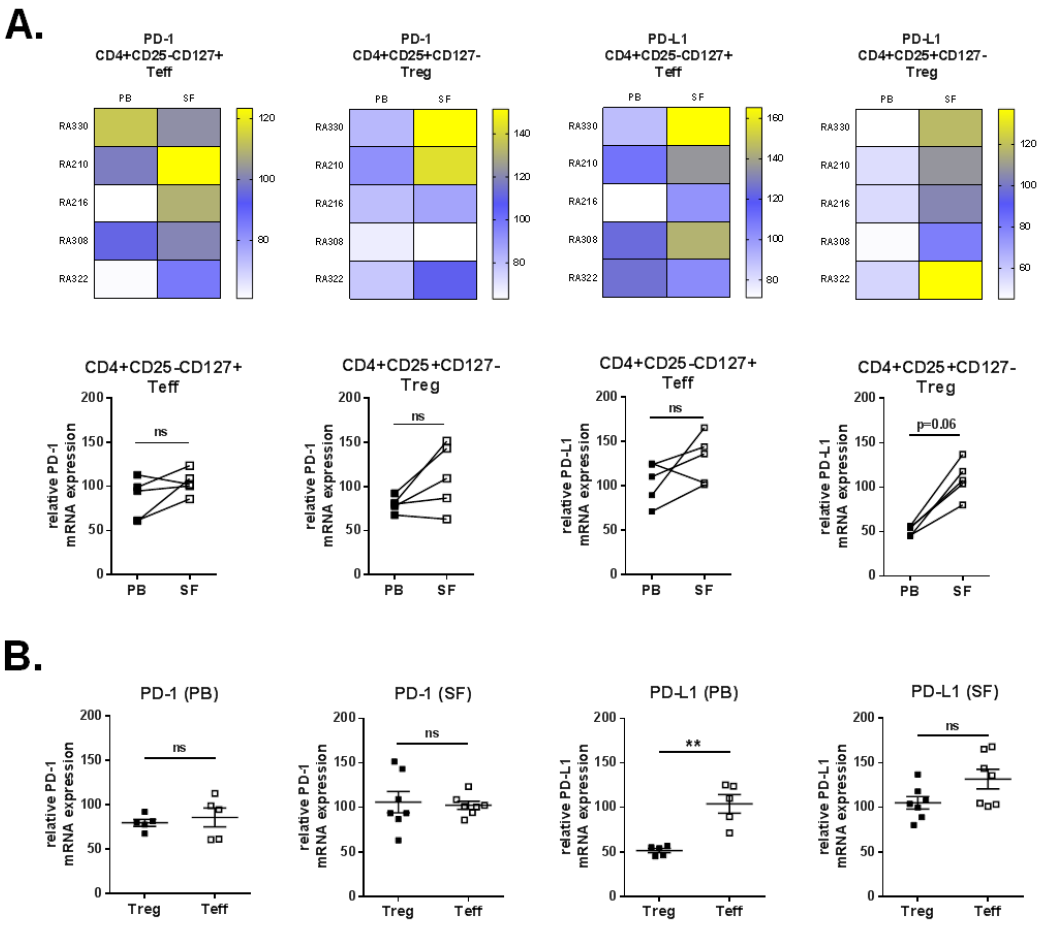

Figure 3.4 PD-1 and PD-L1 mRNA expression of memory (CD45RO+) Teff and Treg CD4+ T cell subsets from the paired PB and SF of patients with RA.
(A) The heatmap and the associated graphs represent the results from the gene expression array and show the relative expression levels of *PDCD1* (PD-1) and *CD274* (PD-L1) genes in the Teff and Treg cell subsets from the PB and SF of and RA patients (n=5). (B) Expression levels of PD-1 and PD-L1 in Treg and Teff cells from the PB (n=5) and SF (n=7) of patients with RA. Data in (A) were analysed by Wilcoxon matched-pairs signed-rank test while data in (B) were analysed by Mann-Whitney test. *p < 0.05, **p < 0.01 and ***p < 0.001. The demographics of the patients used for this analysis can be found here: (Appendix: Table 1).

3.2.3 Gene expression analysis for PD-1 and PD-L1 in HC PB, RA PB and RA SF CD14+ monocytes from RA patients

Next, the expression of *PDCD1* (PD-1) and *CD274* (PD-L1) was evaluated by analysing a previously generated gene expression array performed in HC PB, RA PB and RA SF-derived CD14+ monocytes (250). No significant differences were found in PD-1 expression between HC PB monocytes (HC PBM), RA PB monocytes (RA PBM) and RA SF monocytes (RA SFM) (Figure 3.5A). PD-1 expression was slightly higher in RA PBM compared to HC PBM and further increased in 6 of 9 RA SFM as compared to paired RA PBM but the observed differences were not statistically significant.

Next, PD-L1 expression was analysed between HC PBM and RA PBM and no difference was found. On the contrary, PD-L1 was significantly upregulated in 9 out of 9 RA SFM as compared to paired RA PBM and further increased compared to HC PBM (Figure 3.5B).

These data indicate that CD14+ monocytes deriving from the chronic proinflammatory environment found in the inflamed RA joint have high PD-L1 gene expression and suggests that RA SFM might have the potential to trigger PD-1 activation in PD-1+ cells within the synovial fluid microenvironment.

Figure 3.5

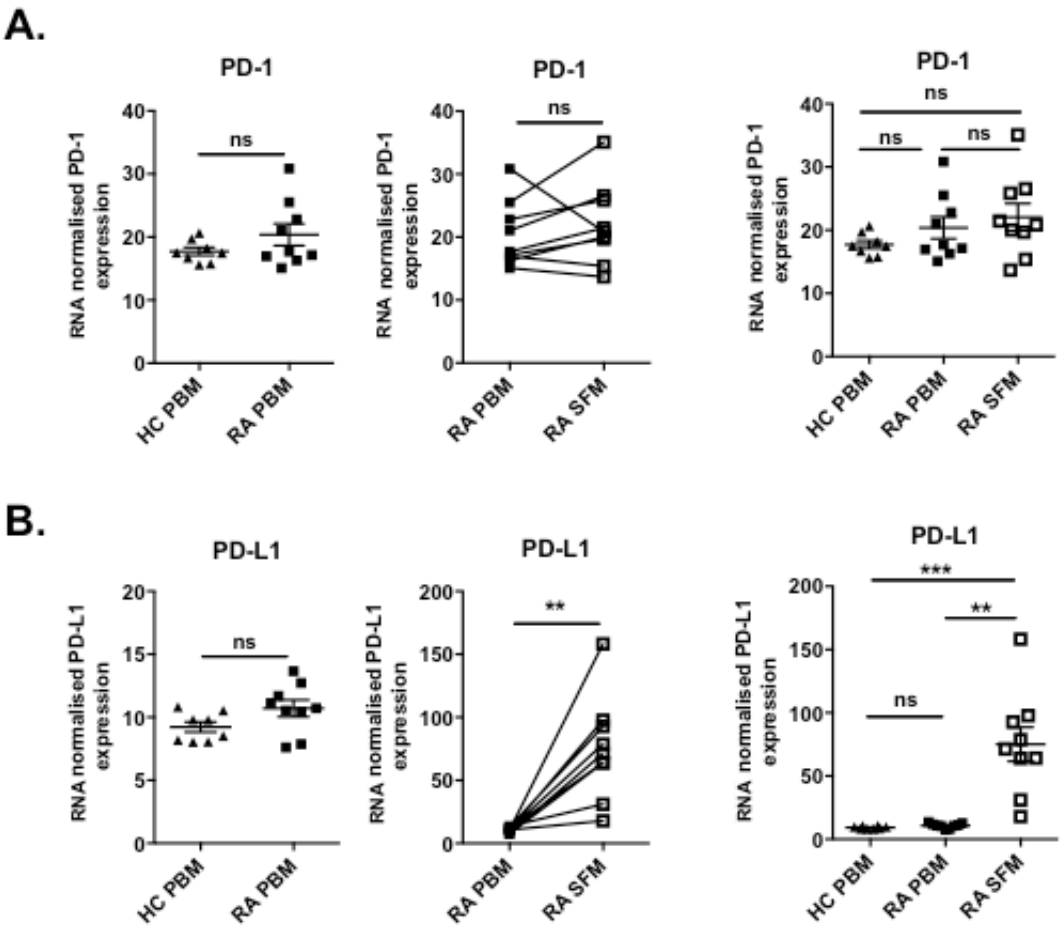

Figure 3.5 PD-1 and PD-L1 mRNA expression in HC PB, RA PB and RA SF-derived CD14+ monocytes.
(A-B) Expression levels of PD-1 and PD-L1 in CD14+ monocytes obtained from healthy PB (n=8) and paired PB and SF from patients with RA (n=7). Data were analysed by Mann Whitney test for HC PBM versus RA PBM or SFM and by Wilcoxon matched-pairs signed-rank test for RA PBM versus RA SFM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. The demographics for the patients used for this analysis can be found here: (250)

3.2.4 Flow cytometry analysis of PD-1 and PD-L1 expression in cells subsets within PBMC and SFMC from RA and PsA patients

So far the analysis performed on three different gene expression arrays have shown that *PDCD1* (PD-1) and *CD274* (PD-L1) genes are differentially expressed within different cell populations and between RA patients and healthy controls. Specifically, PD-1 and PD-L1 are significantly higher in PB memory Teff and Treg cells as compared to PB naïve cells but no differences are found when comparing RA cells and HC cells in any of the 4 populations tested. Both PD-1 and PD-L1 are upregulated in RA SF Teff and Treg as compared to RA PB cells. Finally, PD-L1, but not PD-1, is significantly increased in RA SF-derived monocytes as compared to RA PB and HC PB monocytes.

The next experiments were performed to investigate the cell surface expression of PD-1 and PD-L1 in different cell subsets from both the blood and synovial fluid of patients with RA. This investigation was further extended to samples from PsA patients.

In a first set of experiments, paired PB- and SF-derived mononuclear cells (PBMC and SFMC) were isolated from patients with RA or PsA and incubated *ex vivo* with the following surface markers: CD3, CD8, PD-1 and PD-L1. Fluorescence Minus 2 (FM2) staining for both PBMC and SFMC was used as control and the percentages of PD-1+ or PD-L1+ cells were evaluated within the CD3+CD8+ and CD3+CD8- T cell populations. The gating strategy and the percentage of PD-1+ positive cells in the PB and SF for a representative RA is shown in Figure 3.6. This initial gating strategy and analysis was performed on n=5 RA and n=5 PsA samples with the main aim to confirm literature data for RA and to perform a preliminary investigation for PsA.

A second staining panel was designed and applied to all the subsequent samples analysed in order to obtain more informative data. In the second gating strategy the percentage of PD-1+ or PD-L1+ cells was evaluated within the CD14-CD3+CD8+ and CD14-CD3+CD4+ T cell populations as well as within the CD14+ monocytes population. The gating strategy and the percentage of PD-1+ cells in both PB- and SF-derived CD4+ and CD8+ T cells for two representative stainings (1 PsA and 1 RA) is shown in Figure 3.7A and 3.7B.

The frequencies of PD-1+ cells were significantly increased within SF CD4+ T cells (identified either by CD3+CD8- cells or CD14-CD3+CD4+ cells) in 10 of 10 RA and in 11 of 11 PsA patients as compared to PB (Figure 3.8A and 3.8B).

In addition, increased frequencies of PD-1+ cells were also found within the SF CD8+ T cell compartment in both RA and PsA.

Next, the frequencies of PB- and SF-derived PD-1+ cells within CD4+ T cells and CD8+ T cells were compared between RA and PsA patients (Figure 3.9). For this investigation unpaired RA or PsA samples (SF-only, or PB-only) were also included in the overall analysis. Data show that no significant differences were found between RA and PsA in both the CD4+ and CD8+ T cell subsets (Figure 3.9A and 3.9B).

Figure 3.6

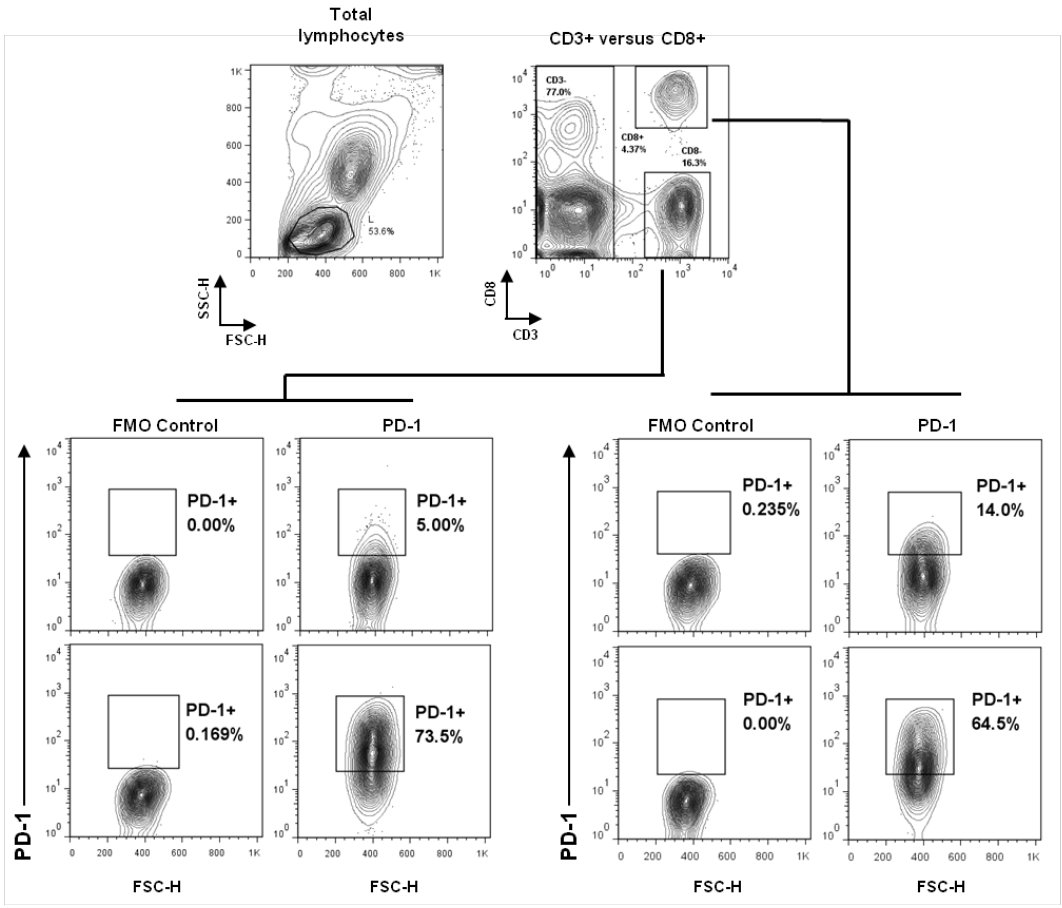

Figure 3.6 Gating strategy for the analysis of PD-1+ cell frequencies in PBMC and SFMC.
Contour plots showing the gating strategy (I) for PD-1 analysis. Cells were gated visually as lymphocytes followed by CD3+ versus CD8+ cells. The percentage of PD-1+ cells was determined within the CD3+CD8+ and CD3+CD8- subsets. The fluorescence minus one control (FMO) was obtained by omitting the PD-1-specific antibody from the antibody staining cocktail. Representative plots for one RA donor are shown.

Figure 3.7

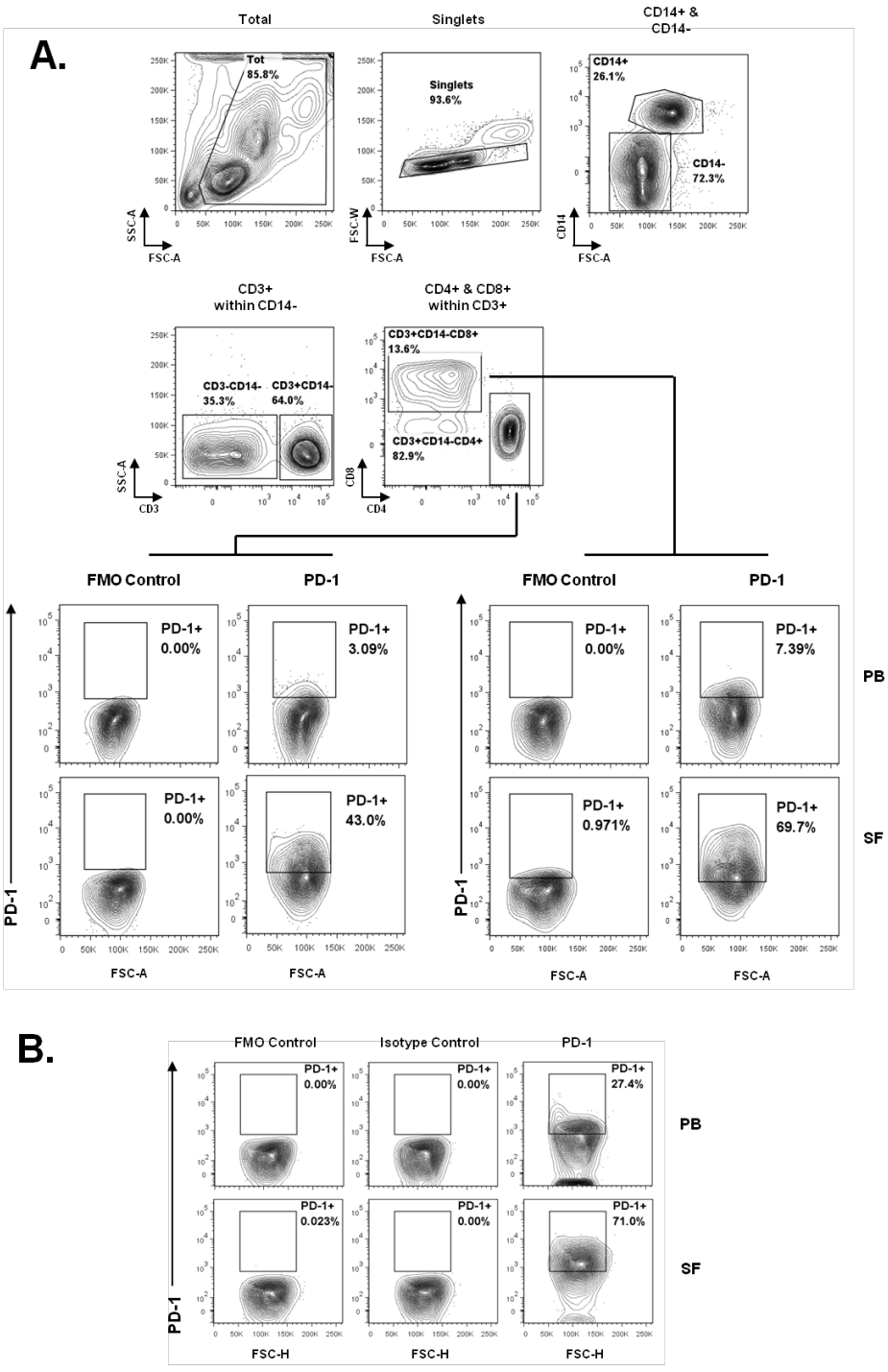

Figure 3.7 Gating strategy for the analysis of PD-1+ cell frequencies in PBMC and SFMC.
Cells were gated as total cells, singlets and CD14+/CD14- cells. CD14- cells were gated as CD3+ and CD3- cells and CD4+ or CD8+ T cells were identified within the CD3+ population. The percentage of PD-1+ cells was determined within the CD4+ and CD8+ subsets. Contour plots of PB and SF cells of one PsA donor (A). For (B) FMO and isotype control of one RA sample are shown.

Figure 3.8

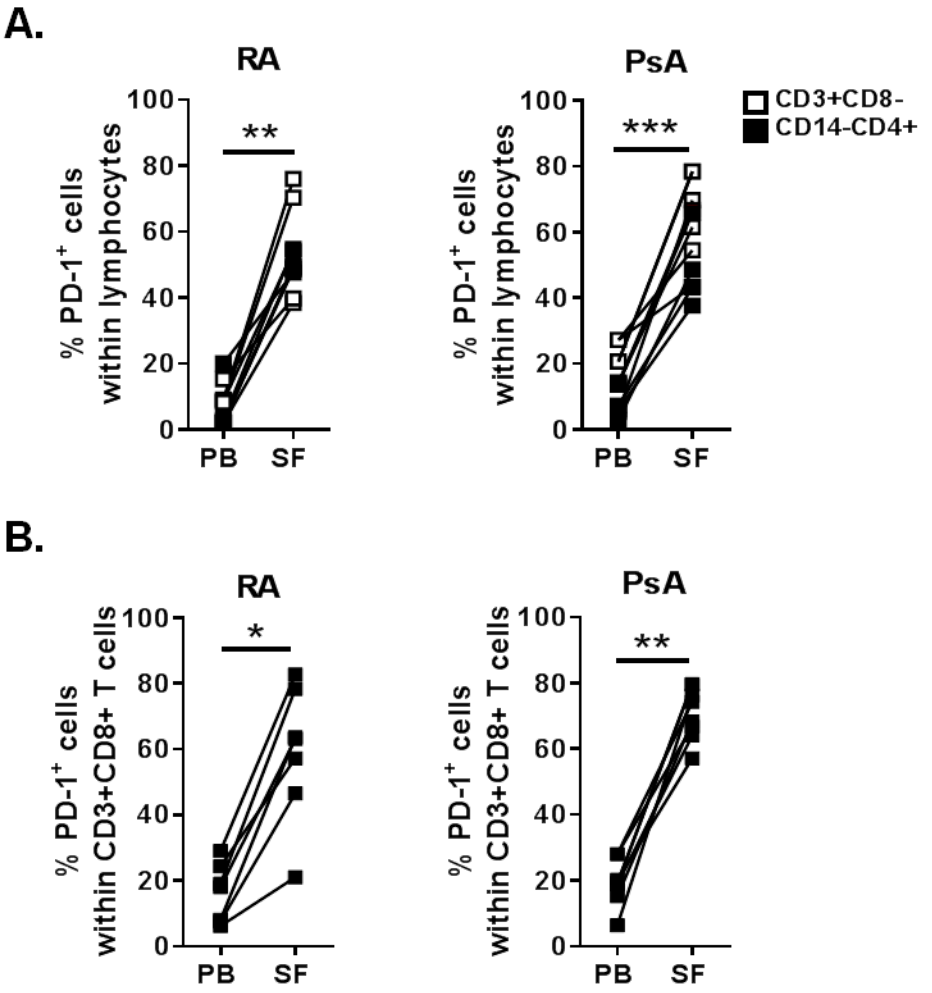

Figure 3.8 PD-1+ T cell frequencies are increased in RA and PsA SF compared to PB. Frequencies of PD-1+ T cells were analysed ex vivo by flow cytometry in PBMC and SFMC from RA and PsA patients. (A) Cumulative data showing percentage of PD-1+ cells within CD3+CD8- or CD3+CD14-CD4+ (RA n=10; PsA n=11) PB and SF cell populations. (B) Cumulative data showing percentage of PD-1+ cells within CD3+CD8+ (RA n=7; PsA n=8) PB and SF cell populations. Data were analysed by Wilcoxon matched-pairs signed-rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 3.9

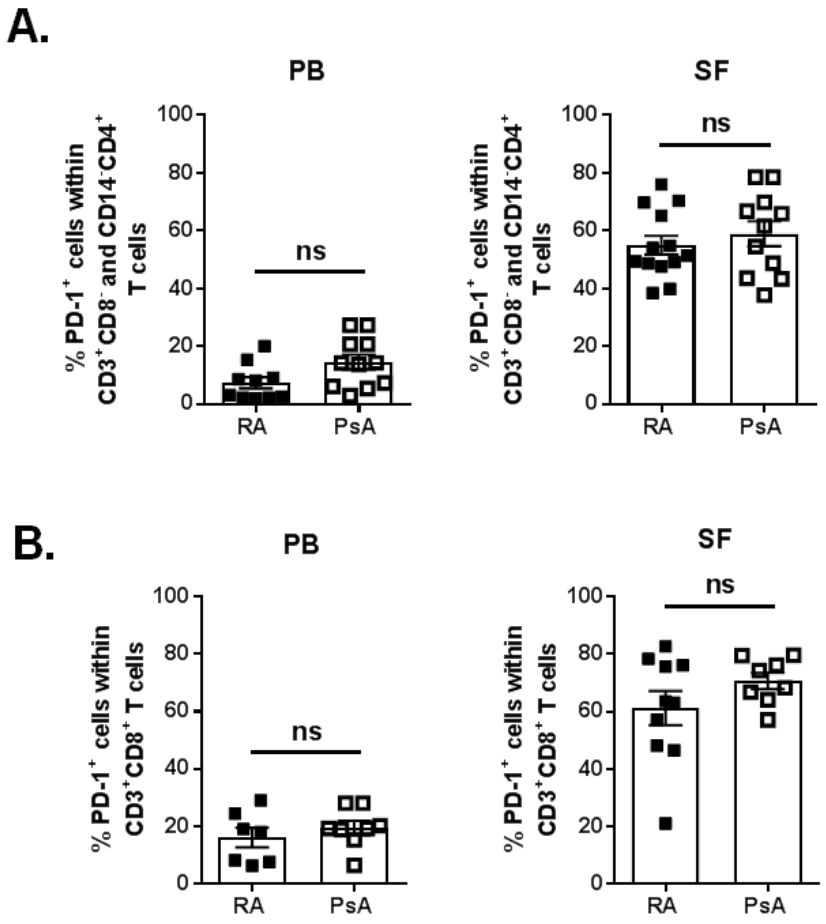

Figure 3.9 PD-1+ T cell frequencies are similar between RA and PsA.
Frequencies of PD-1+ T cells were analysed *ex vivo* by flow cytometry in RA and PsA PBMC and SFMC and compared between the two diseases. (A) Cumulative data showing the percentage of PD-1+ cells within CD3+CD8- or CD3+CD14-CD4+ PB (RA n=10; PsA n=11) and SF (RA n=13; PsA n=11) cell populations. (B) Cumulative data showing percentage of PD-1+ cells within CD3+CD8+ PB (RA n=7; PsA n=8) and SF (RA n=10; PsA n=8) cell populations. Data were analysed by Wilcoxon matched-pairs signed rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Mean ± SEM is shown.

Next, the frequencies of PD-L1+ cells amongst T cells in paired PB and SF were analysed. Increased percentages of PD-L1+ cells were found in 6 of 6 RA patients and in 4 of 5 PsA patients within SF CD4+ T cells (identified either by CD3+CD8- cells or CD14-CD3+CD4+ cells) as compared to PB but only RA data were statistically significant (Figure 3.10A). No significant differences were found in the percentages of PD-L1+ cells within the SF CD8+ T cell compartment in both RA and PsA (Figure 3.10B). The observed statistical differences between RA and PsA likely reflect the low number of patients available when this analysis was performed. The frequencies of PB and SF-derived PD-L1+ cells within CD4+ and CD8+ T cells were compared between patients with RA or PsA and no significant differences were found (Figure 3.11).

PD-1 expression was further analysed by flow cytometry in RA and PsA PB- and SF-derived Teff and Treg cells. These experiments were performed to confirm data from the gene expression array (section 3.2.2, Figure 3.4). Teff and Treg cells were identified as CD4+CD25-CD127+ and CD4+CD25+CD127-, respectively. Figure 3.12A shows the gating strategy and the percentages of Teff and Treg cells found in the PB and SF of a representative RA sample. In line with published data (338-344), Treg cells were increased in the synovial fluid as compared to peripheral blood. Figure 3.12B shows a consistent increase of Treg cells percentage in both RA and PsA SF samples (paired data) and an overall Treg cell percentage of ~14% for inflammatory arthritis (IA) SF as compared to ~6% for IA PB (unpaired data) (Figure 3.12B). Increased percentages of PD-1+ cells were found in 3 of 3 RA and in 3 of 3 PsA patients within SF Teff and Treg cells compared to paired PB (Figure 3.13A). A second analysis performed including unpaired samples, showed significantly increased percentages of PD-1+ cells within both SF-derived Teff (~48% PD-1+ cells)

and Treg (~40% PD-1+ cells) cells as compared to PB-derived Teff (~5% PD-1+ cells) and Treg (~7% PD-1+ cells) cells (Figure 3.13B). These data are in line with the gene expression analysis described in section 3.2.2 and demonstrate that the increase in PD-1 gene expression observed in RA-SF is coupled with increase of the PD-1 protein at the cell surface level. Furthermore, these data indicate that a similar increase at the cell surface level is also detected for PsA.

Finally, the frequencies of PD-1+ and PD-L1+ cells amongst CD14+ monocytes in PB and paired SF were analysed. No significant differences in the percentages of PD-1+ cells were found within IA SF CD14+ cells as compared to PB. Overall, ~68% of CD14+PD-1+ cells were detected in both IA PB and SF (Figure 3.14A). This is in line with the results from the gene expression array described in figure 3.5A showing no significant differences in PD-1 expression between RA PB- and SF-derived CD14+ monocytes. On the contrary, a significant decrease in the percentages of PD-L1+ cells was detected within IA SF-derived CD14+ cells as compared to SF (~48% of CD14+PD-L1+ cells in the PB compared to ~20% in the SF) (Figure3.14B). This is in contradiction with the data obtained from a different set of RA patients and described in Figure 3.5B which showed a significant increase in PD-L1 gene expression in SF-derived monocytes as compared to PB-derived monocytes.

Figure 3.10

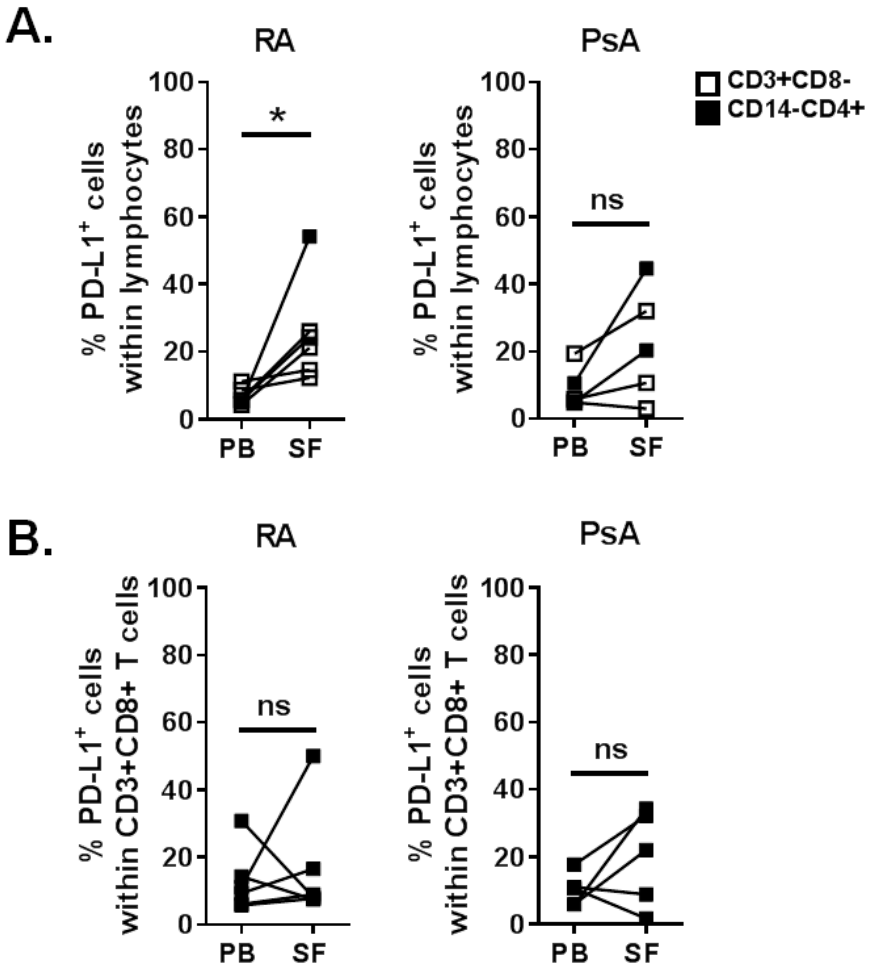

Figure 3.10 PD-L1+ T cell frequencies are not consistently increased in RA and PsA SF compared to PB.
Frequencies of PD-L1+ T cells were analysed *ex vivo* by flow cytometry in PBMC and SFMC from RA and PsA patients. (A) Percentage of PD-L1+ cells within CD3+CD8- or CD3+CD14-CD4+ (RA n=6; PsA n=5) PB and SF cell populations. (B) Percentage of PD-L1+ cells within CD3+CD8+ (RA n=6; PsA n=7) PB and SF cell populations. Data were analysed by Wilcoxon matched-pairs signed-rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 3.11

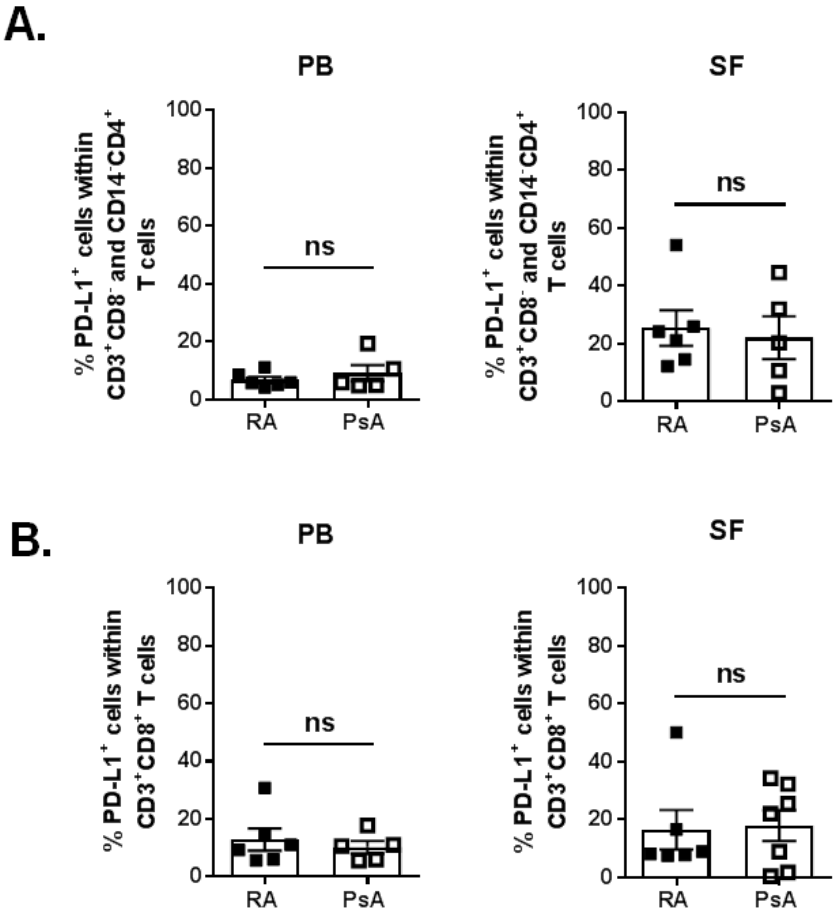

Figure 3.11 PD-L1+ T cell frequencies are similar between RA and PsA.
Frequencies of PD-L1+ T cells were analysed *ex vivo* by flow cytometry in RA and PsA PBMC and SFMC and compared between the two diseases. (A) Percentage of PD-L1+ cells within CD3+CD8- or CD3+CD14-CD4+ PB and SF (RA n=6; PsA n=5) cell populations. (B) Cumulative data showing percentage of PD-1+ cells within CD3+CD8+ PB (RA n=6; PsA n=5) and SF (RA n=6; PsA n=7) cell populations. Data were analysed by Wilcoxon matched-pairs signed rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Mean ± SEM is shown.

Figure 3.12

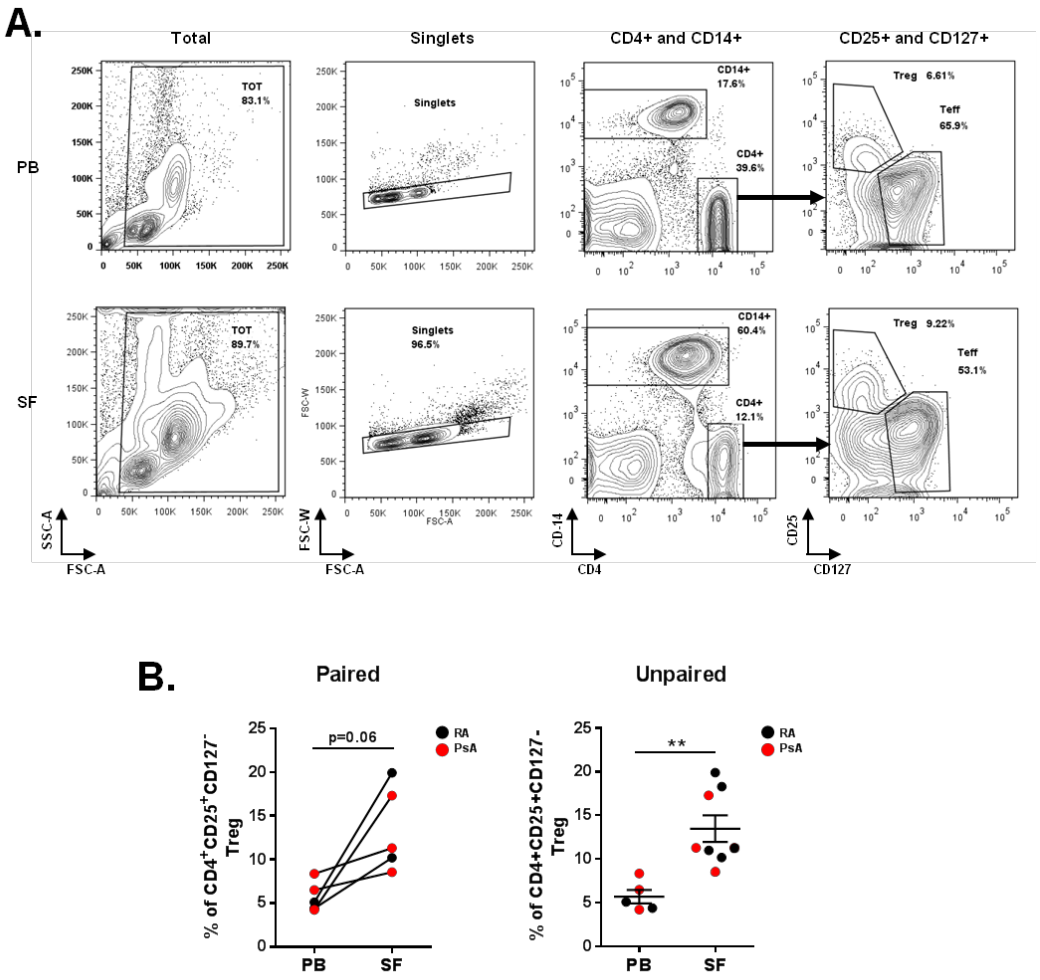

Figure 3.12 Treg cells frequencies in RA and PsA SF are increased compared to PB. Frequencies of CD4+CD25+CD127- cells were analysed *ex vivo* by flow cytometry in PBMC and SFMC from RA and PsA patients. (A) Contour plots of a representative RA sample (PB and SF) showing the gating strategy for the identification of CD4+ Treg cells. Cells were gated as total cells followed by single cells and CD14+ vs. CD4+ cells. CD4+ cells were plotted as CD127 vs. CD25 cells. (B). Percentage of Treg cells in RA and PsA PB and SF (Paired analysis: RA n=2; PsA n=3; Unpaired analysis: RA PB n=2, RA SF n=5; PsA PB and SF n=3) Data were analysed by Wilcoxon matched-pairs signed-rank test and Mann-Whitney test. *p < 0.05, **p < 0.01 and ***p < 0.001. Mean ± SEM is shown.

Figure 3.13

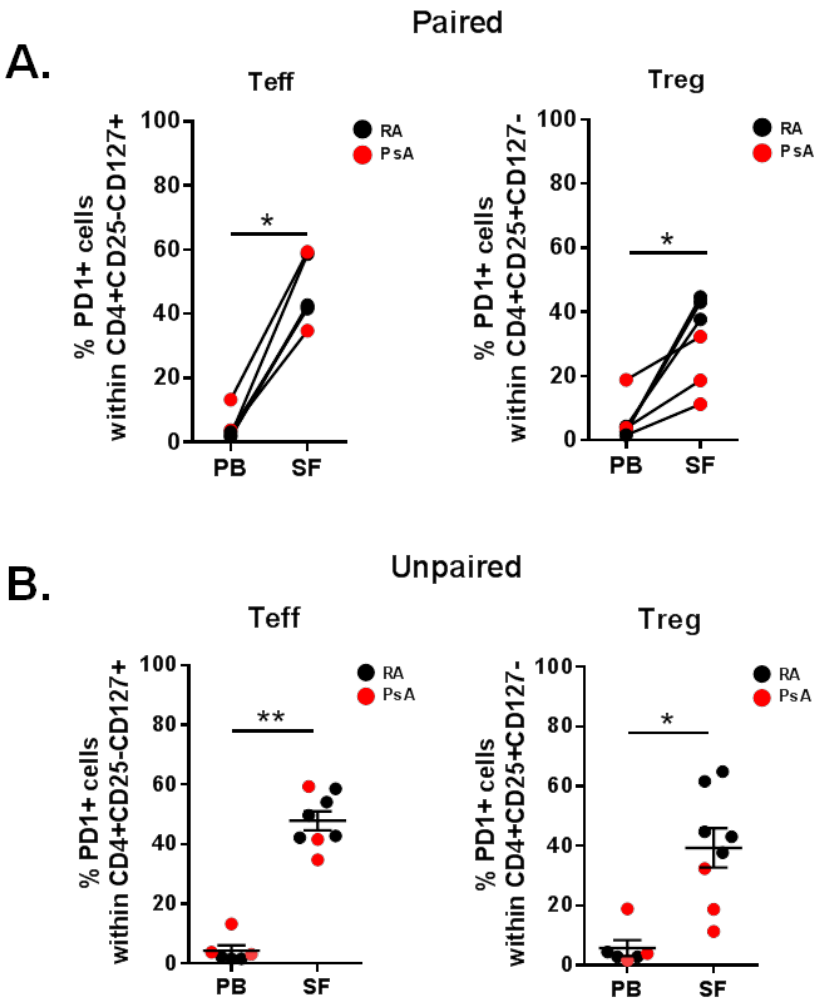

Figure 3.13 PD-1+ T cell frequencies are increased in RA and PsA SF-derived Teff and Treg cells compared to PB cells.
Frequencies of PD-1+ T cells were analysed *ex vivo* by flow cytometry in PBMC and SFMC from RA and PsA patients. (A) Percentage of PD-1+ cells within Teff and Treg (RA n=3; PsA n=3) PB and SF cell populations (paired analysis). (B) Percentage of PD-1+ cells within Teff and Treg (RA PB n=3, RA SF n=5; PsA PB n=3, PsA SF n=3) PB and SF cell populations. Data were analysed by Wilcoxon matched-pairs signed-rank test (A) and by Mann-Whitney test (B). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Mean ± SEM is shown.

Figure 3.14

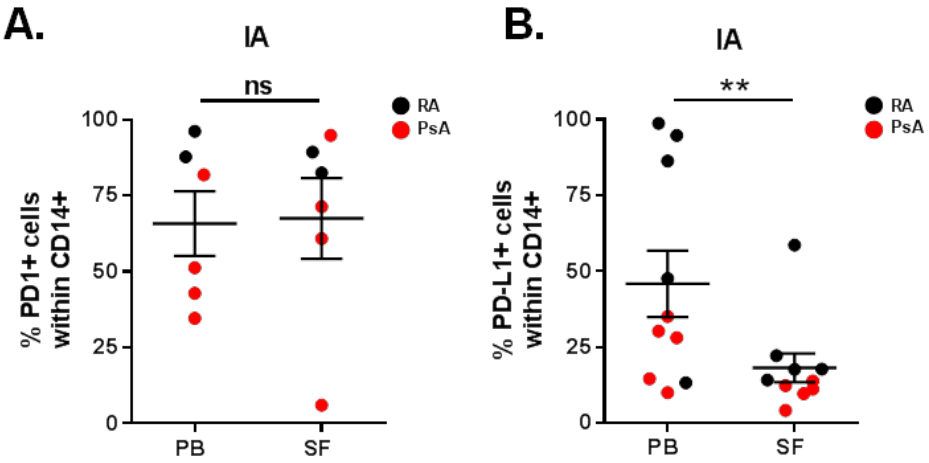

Figure 3.14 PD-1+ and PD-L1+ cell frequencies within CD14+ monocytes are not increased in RA and PsA SF-derived cells compared to PB.
Frequencies of PD-L1+ cells were analysed *ex vivo* by flow cytometry in PBMC and SFMC from RA and PsA patients. (A) Percentage of PD-1+ cells within RA and PsA PB and SF CD14+ monocytes (RA n=2; PsA n=4). (B) Percentage of PD-L1+ cells within RA and PsA PB and SF CD14+ monocytes (RA n=5; PsA n=5). Data were analysed by Wilcoxon matched-pairs signed-rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Mean ± SEM is shown.

3.3 Discussion

The data presented in this chapter provide new findings regarding the levels of expression of PD-1 and PD-L1 within different cell subsets in both RA and PsA. Gene expression analysis performed on PB-derived Teff and Treg cells found no differences in PD-1 and PD-L1 expression between RA patients and healthy controls. A significant enrichment of PD-1 and PD-L1 expression was detected only within the memory cell compartment of both HC and RA, as compared to naïve T cells. On the contrary, gene expression analysis performed on RA PB- and RA SF-derived cells showed that the expression of both PD-1 and the PD-L1 was increased in SF Teff and Treg cells as compared to PB cells. In line with these data, higher frequencies of PD-1+ cells were found within Teff and Treg cells in both RA and PsA SF as compared to PB. PD-1+ cell frequencies within bulk CD3+CD4+ and CD3+CD8+ T cells were also increased in both RA and PsA SF as compared to PB but no difference was found between the two diseases. No significant differences, except for RA CD4+ T cells, were found in the frequencies of PD-L1+ T cells between PB and SF. PD-1 expression, both at gene and protein level, was comparable between RA PB and RA SF CD14+ cells while a significant enrichment in PD-L1 gene expression was detected in RA SF monocytes as compared to HC and RA PB-derived monocytes although this was not confirmed by flow cytometry in a different set of patients.

In the literature, several studies performed in mouse models of autoimmune disease indicate that PD-1 and its ligand PD-L1 are necessary to control activation and cytokine production of self-reactive T cells. In the NOD mouse model of diabetes, loss or blockade of either PD-1 or PD-L1 leads to exacerbated disease and increased production of proinflammatory cytokines by T cells (210, 345). In C57BL/6 mice with

active experimental autoimmune encephalomyelitis (EAE), PD-1 and its ligands PD-L1 and PD-L2, are highly expressed on cellular infiltrates within the meninges (110) and blockade of PD-1 and PD-L1 leads to accelerated disease severity, increased presence of reactive T cells and enhanced antibody production (110, 213). Further evidence for the critical role of PD-1 in immune regulation can also be found in disruption studies demonstrating that in C57BL/6 and BALB/c mice, the lack of PD-1 leads to development of lupus-like autoimmune proliferative arthritis (57) and severe autoimmune dilated cardiomyopathy (58).

As a result of these findings, several studies have investigated whether inducing or overexpressing PD-L1 to trigger PD-1-mediate signalling may be beneficial in treating different types of autoimmune diseases. Hirata *et al.* showed that mouse dendritic cells (DCs) genetically engineered to overexpress PD-L1 and myelin oligodendrocyte glycoprotein (MOG) significantly reduced T cell responses to MOG, cell infiltration into the spinal cord, and the overall severity of MOG peptide-induced EAE (346). Similarly, Ding *et al.* found that in autoimmune BXSB mice, which have inflammatory features similar to human systemic lupus erythematosus (SLE), the use of a recombinant adenovirus expressing the full-length mouse PD-L1 was able to delay the onset of proteinuria, reduce IgG autoantibody production and negatively modulate cell proliferation resulting in amelioration of lupus nephritis (347). Finally, Schreniner *et al.* demonstrated that IFN-β, an immune-modulatory treatment for multiple sclerosis (MS), was able to upregulate PD-L1 on APC both *in vitro* and in MS patients *in vivo* inhibiting autologous CD4+ T-cell activation. Importantly, neutralisation of PD-L1 on monocytes or monocyte-derived DCs was shown to increase the production of inflammatory cytokines IFN-γ and IL-2 and to induce T-cell proliferation (348). These findings suggest that the PD-1:PD-L1 pathway is

important to control T cell activation and has the potential to become a new therapeutic target in different autoimmune diseases.

In human RA limited data exist regarding the expression levels of PD-1 and PD-L1 in cell phenotypes other than bulk CD4+ T cells while in PsA, data are practically non-existent. Hatachi *et al.* have shown that PD-1+CD4+ T cells are increased in the synovial fluid of RA patients treated with disease-modifying anti-rheumatic drugs (DMARDs) or prednisolone as compared to HC and OA patients (323). In line with these findings, Wan *et al.* also found higher percentages of PD-1+CD4+ T cells in the SF of RA patients not receiving treatment as compared to OA and healthy controls (324).

A study performed on RA patients treated with either DMARD or anti-TNF medication confirmed that CD4+PD-1+ T cells are increased in the SF as compared to OA controls (216). The study further demonstrated that PD-1 is expressed in RA synovial tissue but not in HC and OA tissue and that PD-1 ligands PD-L1 and PD-L2 are expressed in both RA and OA but not in HC tissue (216). Finally, work from Rao *et al.* recently identified a new PD-1+CD4+ subset of T cells in the joints and blood of seropositive RA patients. This subset, named peripheral helper T cells (T_{HP}), is phenotypically characterised as PD-1^{hi}CXCR5-CD4+ and expresses a variety of chemokine receptors such as CCR2, CX3CR1, and CCR5 (349) (see chapter 4 for a description of T_{HP} functional characterisation).

Evidence supporting a possible contribution of PD-1 in modulating T cell responses in PsA can be found in studies on human and mouse psoriasis. Interestingly, about 15% of psoriasis patients also have undiagnosed PsA. The manifestation of psoriasis usually precedes that of arthritis by an average of 10 years and PsA is

believed to develop in about 25-30% of patients affected by psoriasis (292, 293). Kim *et al.* found that PD-1 is overexpressed on IL-17+ T cell in the inflamed skin tissue of psoriasis patients and in mice with imiquimod (IMQ)-induced psoriasis. Furthermore, in IMQ-treated mice, the use of a PD-L1fc fusion protein to trigger PD-1 activation reduced psoriatic inflammation when given alone and enhanced the therapeutic effect of anti-p40 antibodies when given in combination (350). Similarly, Imai *et al.* compared skin responses of PD-1–deficient (PD-1 KO) mice and wild-type controls in an (IMQ)-induced murine model of psoriasis and demonstrated that PD-1 KO mice showed severe epidermal hyperplasia, greater neutrophils infiltration, and higher expression of the cytokine Th17 (351).

Thus far, only one study has investigated PD-1 expression in PsA. Peled *et al.* have shown that PD-1+CD3+ T cells are increased in the peripheral blood of PsA patients as compared to healthy volunteers and that the levels of PD-1 expression inversely correlate with the number of tender and swollen joints, but not with the psoriasis area and severity index (PASI) or with the C-reactive protein (CRP) levels (352).

The data presented in this chapter confirm previous findings from the literature and further extend our knowledge on the expression levels of PD-1 and PD-L1 in both RA and PsA. The analysis of PD-1 and PD-L1 gene expression in CD4+CD45RA+ or CD4+CD45RO+ Teff and Treg cells from the PB revealed no statistically significant differences between RA patients and healthy controls. Significant differences were only found in the expression of PD-1 and PD-L1 between naïve and memory cells suggesting that these molecules might be involved in T cell self-regulation during secondary immune responses triggered by re-exposure to recurrent antigens. Data analysis from a second gene expression array, performed on RA CD4+CD45RO+

cells, demonstrated that the expression of PD-1 and PD-L1 was increased in SF Teff and Treg cells as compared to PB cells although the results were not statistically significant. PD-1 data are in line with work from Hatachi *et al.*, which described that the majority of PD-1+CD4+ cells found in the RA SF were also CD45RO+ (323). Importantly, flow cytometry experiments performed on RA and PsA samples confirmed that the percentages of PD-1+ cells were increased within RA SF CD4+ Teff and Treg cells as compared to PB cells and further showed that this was also the case for PsA. Further experiments presented in this chapter demonstrated that PD-1+ cells are also significantly increased within SF-derived CD3+CD4+ and CD3+CD8+ T cells as compared to PB. This increase can be observed both in RA and PsA patients suggesting a similar role for PD-1 in these two different arthrides.

These results are important for two reasons. Firstly, they confirm and extend previous studies, which focused only on bulk CD4+ T cells from RA patients (216, 323, 324). Secondly, they characterise PD-1 expression in PsA showing that the receptor appears to be equally expressed between the two diseases within specific cell populations both in the blood and synovial fluid.

Interestingly, the analysis performed on the second gene expression array also suggests that PD-L1 might be upregulated in Teff and Treg cells from the synovial fluid of RA patients. This has not been described before and its biological significance is unclear. During this thesis, scarcity in sample availability precluded a comprehensive study on the surface levels of PD-L1 in RA and PsA-derived Treg and Teff cells. Furthermore, considerable variation in PD-L1 expression was found amongst RA and PsA samples in SF-derived CD4+ and CD8+ T cells as compared to PB. Hence, further experiments are required before conclusive statements can be made regarding PD-L1 expression in RA and PsA.

Nevertheless, it is important to point out that PD-L1+ T cells might have very distinct and important roles within the inflamed joint. PD-L1+ T cells could use the ligand to trigger PD-1 activation on other cells in proximity modulating their proliferation and cytokine production. PD-L1+ T cells might also have a role in regulating Treg cells function skewing other T cells towards a Treg-like phenotype. This hypothesis is supported by work from Francisco *et al.*, which showed that PD-L1 regulates development of Treg and sustains FoxP3 expression enhancing Treg suppressive capacities (98) (see chapter 5 and chapter 6 for further details on the role of PD-L1 and Treg cells).

Finally, the presence of PD-L1 on SF-derived cells could have a role in modulating chronic T cell responses. This is interesting because although no polymorphisms in the CD274 gene (PD-L1) have been linked to human autoimmune diseases yet, Dong *et al.* described increased autoantibodies against PD-L1 in the serum of RA patients as compared to healthy volunteers and demonstrated that immobilized antibodies towards PD-L1 were capable of stimulating CD4+ T cell proliferation *in vitro* suggesting a possible bidirectional signalling role of PD-L1 in T cell costimulation (353).

Currently, it is unclear why T cell activation persists despite high frequencies of PD-1+ T cells detected within RA and PsA joints. One possible explanation is that the expression of PD-1 costimulatory ligands is insufficient in synovial T cells and macrophages. Three studies investigated PD-L1 cell surface expression on RA SF-derived CD14+ monocytes as compared to PB-derived monocytes. The results from these groups are, however, contradictory with the first two groups reporting increased percentages in RA SF-derived PD-L1+CD14+ cells compared to RA PB (324, 354)

while the third group reporting a lower percentage of PD-L1+CD14+ cells in the SF as compared to PB (216). Analysis from our third array indicates that PD-L1 gene expression is increased in RA SF monocytes as compared to HC and RA PB monocytes. However, flow cytometry analysis performed on a new set of RA and PsA samples could not confirm these results at the cell surface level and in line with work from Raptopoulou *et al.* (216), lower percentages of PD-L1+CD14+ monocytes were detected in the SF as compared to PB.

Some of these differences may be attributed to the variation in patient groups with respect to disease activity and therapy. It is also plausible that when monocytes are taken out of their proinflammatory environment PD-L1 might be internalised limiting the antibody detection power. Interestingly, it was shown by Chang *et al.* that in tumour cells expressing PD-L1, the ligand moved from the surface to the interior of the cell after treatment with anti-PD-L1 blocking antibody for 30 minutes at 37°C, indicating internalisation (355). Further experiments with increased numbers of donors and different reagents might reveal whether PD-L1 is truly increased in SF-derived monocytes.

The next chapter explores the ability of PD-1 ligation to modulate cell proliferation and cytokine production in CD4+ T cells from healthy volunteers and from patients with RA and PsA. Further attention is given to the role of RA and PsA-derived proinflammatory cytokines and to TNF and IL-6 blocking antibodies.

4 Inflammation negatively modulates PD-1-mediated suppression of proliferation of human CD4+ T cells

4.1 Introduction

The PD-1 receptor is a trans-membrane protein and member of the B7 family which plays a critical role in T cell regulation (356). PD-1 is expressed on T cells, where its expression increases within the first 24 hrs of T cell activation and decreases with antigen clearance (69, 91, 357). Upon ligation of PD-1 by its ligands (PD-L1/B7-H1 and PD-L2/B7-DC), T cell responses are downregulated (66, 95, 345). Signalling through PD-1 leads to inhibition of the phosphatidylinositol 3-kinase (PI3K) resulting in reduced Akt phosphorylation and reduced expression of transcription factors Gata3, T-bet and Eomes (36, 86). The overall effect of PD-1 ligation is a reduced phosphorylation of TCR signalling molecules leading to decreased T cell proliferation and cytokine production (60, 61, 345). In mice, it has been documented that disruption of the PD-1 gene (*pdcd1*) leads to lupus-like syndrome, proliferative arthritis, diabetes, autoimmune cardiomyopathy and increased susceptibility to collagen-induced arthritis (CIA) (57, 58, 209, 218, 358). In humans, polymorphisms in the *PDCD1* gene have been associated with susceptibility to rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes mellitus (236, 359-365).

Several investigators, including our own team (chapter 3), have shown that frequencies of PD-1+CD4+ T cells are increased in RA synovial fluid (SF) compared to RA peripheral blood (PB) (216, 323, 324). However, despite high levels of this inhibitory receptor at the site of inflammation, the immune system seems unable to regulate persistent T cell activation and cytokine production. Such evidence poses the

question as to whether the PD-1 pathway is impaired in the inflamed joint. Thus far, only limited data exist regarding PD-1 function in human inflammatory arthritis. An indication of a defective PD-1 pathway in RA comes from a study reporting that PD-1 ligation inhibited cell proliferation and IFN-γ production by CD4+ T cells from peripheral blood of RA patients but that synovial fluid CD4+ T cells required higher concentrations of the ligand to achieve similar levels of inhibition. The same study also reported that peripheral blood CD4+ T cells from healthy controls (HC), RA and OA donors display comparable proliferation upon PD-1 ligation (216). While this research was unfolding, it was unclear whether PD-1-mediated suppression of proliferation was affected by proinflammatory cytokines typically associated with inflammatory arthritis. Moreover, the few studies investigating PD-1-ligation in inflammatory arthritis mainly focused on RA (216, 366), whereas little was known in the context of psoriatic arthritis (PsA).

This was of particular interest because although PsA and RA are two distinct diseases with serological, genetic, and radiological differences they share a number of common pathological features including systemic chronic inflammation (367). The experiments in this chapter were designed to further investigate the concept of impaired PD-1-mediated regulation in RA, extend it to a different inflammatory joint disease (PsA) and explore whether inflammation influences the ability of the PD-1 pathway to function effectively.

Therefore, the aims of this chapter were the following:

1. Setting up and optimise a reliable assay to test whether PD-1 ligation is able to modulate cell proliferation and cytokine production by HC CD4+ T cells.

2. Investigate whether PD-1 is functional in CD4+ T cells derived from RA and PsA peripheral blood and synovial fluid.
3. Test how different proinflammatory mediators and biologics influence PD-1-mediated T cell suppression of HC CD4+ T cells.

4.2 Results

4.2.1 PD-1 ligation of HC CD4+ T cells

The first aim of this chapter was to set up an assay to examine how PD-1 ligation modulates cell proliferation and cytokine production in the presence of its ligand PD-L1. CD4+ T cells were negatively isolated from the PB of HC donors (purities described in Figure 4.1) and cultured according to previously described protocols (60, 127, 216, 368) using increasing concentrations of either PD-L1fc ligand or IgG1fc control protein. On day 4, cell cultures were pulsed with 0.25 μCi of [^{3}H]-thymidine and cell proliferation was assessed by thymidine uptake after 18 hours, on day 5.

The effect of PD-L1fc on the proliferation of TCR stimulated HC CD4+ T cells is shown in Figure 4.2A and B and is expressed as count per minute (cpm) and suppression of proliferation. Data show that PD-1 ligation in presence of PD-L1 ligand led to a significant and dose-dependent reduction of cell proliferation, whilst no effect was observed in the presence of the appropriate IgG1fc control (Figure 4.2). These initial experiments were performed with low concentrations of PD-L1fc (0, 0.1 and 1 μg/ml) in line with a previous investigation (216). In these experiments the suppression of proliferation was overall ~20% and ~35% in presence of 0.1 and 1 μg/ml of PD-L1fc as compared to the medium only condition (0 μg/ml). Further experiments including a wider range of concentrations of PD-L1fc ligand and IgG1fc control (0, 0.1, 1, 2 and 5 μg/ml) were performed and data confirmed that in HC CD4+ T cells, PD-1 ligation led to a dose-dependent reduction in cell proliferation with highest suppression of ~48% at 5 μg/ml of ligand as compared to the medium only condition (0 μg/ml) (Figure 4.3A and 4.3B).

Next, the ability of PD-1 ligation to modulate IFN-γ production was investigated. IFN-γ is an established readout cytokine known to be negatively modulated following PD-1 ligation (60, 61, 345). Supernatants from HC CD4+ T cell cultured in presence of PD-L1fc were collected at day 5 and tested for the presence of IFN-γ by enzyme-linked immunosorbent assay (ELISA).

Notably, IFN-γ production was also inhibited in a dose-dependent fashion both at low (Figures 4.4A) and high (Figure 4.4B) concentrations of PD-L1fc ligand. Importantly, no decrease in IFN-γ production was detected when HC CD4+ T cells were cultured in presence of the IgG1fc control confirming the specificity of the effect mediated by the PD-L1fc ligand (Figure 4.4A).

The experiments described above indicate that when HC CD4+ T cells are cultured *in vitro* following TCR-only stimulation, the PD-1 receptor is fully functional and capable of negatively regulating cell proliferation and cytokine production.

Figure 4.1

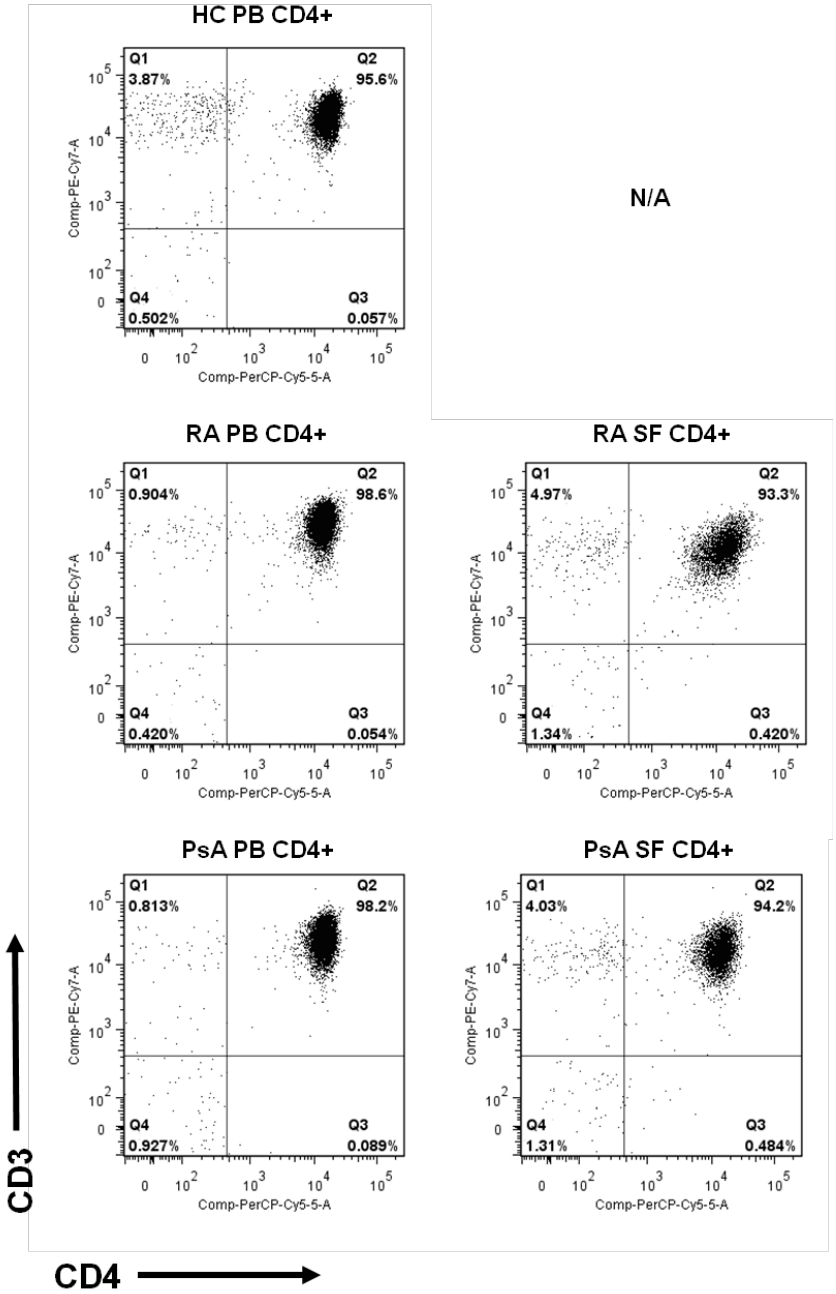

Figure 4.1 Extracellular purity staining of negatively isolated CD4+ T cells.
Representative example of purity stainings of CD4+ T cells isolated from HC PBMC and RA and PsA PBMC and SFMC. In this example cells were stained with PerCP-Cy5.5-labelled CD4 antibody and PE-Cy7-labelled CD3 antibody.

Figure 4.2

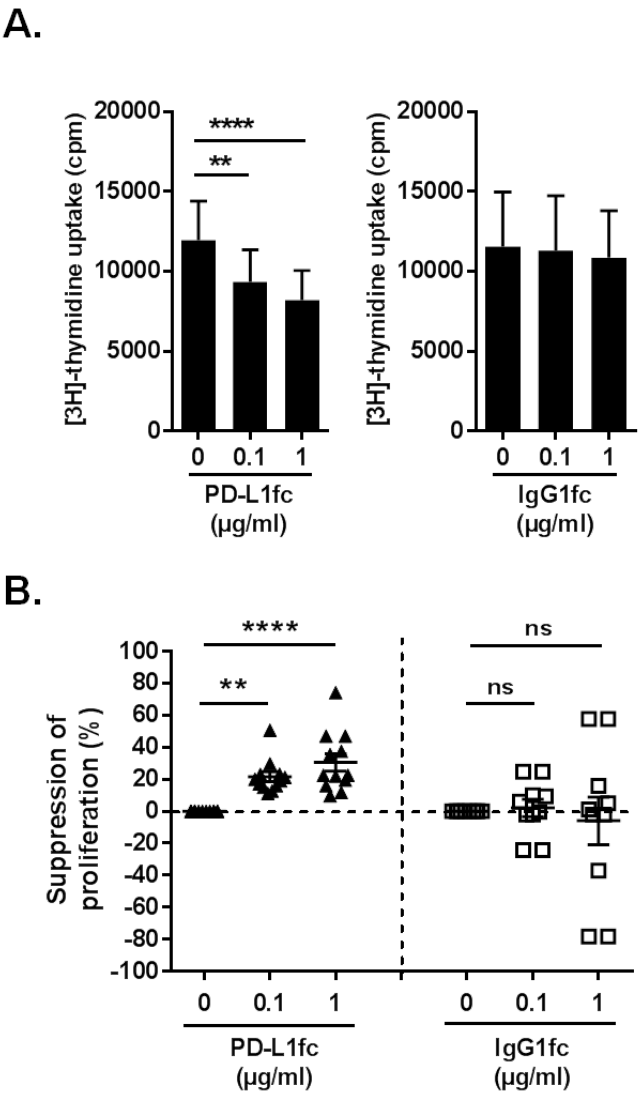

Figure 4.2 PD-1 ligation reduces proliferation of CD4+ T cells from healthy donors.
CD4+ T cells were isolated from HC PBMC and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc/IgG1fc (0, 0.1, 1 µg/ml). Proliferation was assessed on day 5 by [^{3}H]-thymidine incorporation. (A) HC CD4+ T cell proliferation (cpm) and (B) percentage suppression of proliferation following PD-1 ligation by PD-L1fc (n=12) or IgG1fc (n=10). Data in A are shown as the mean ± SEM of n=10-12. Data (A-B) were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.3

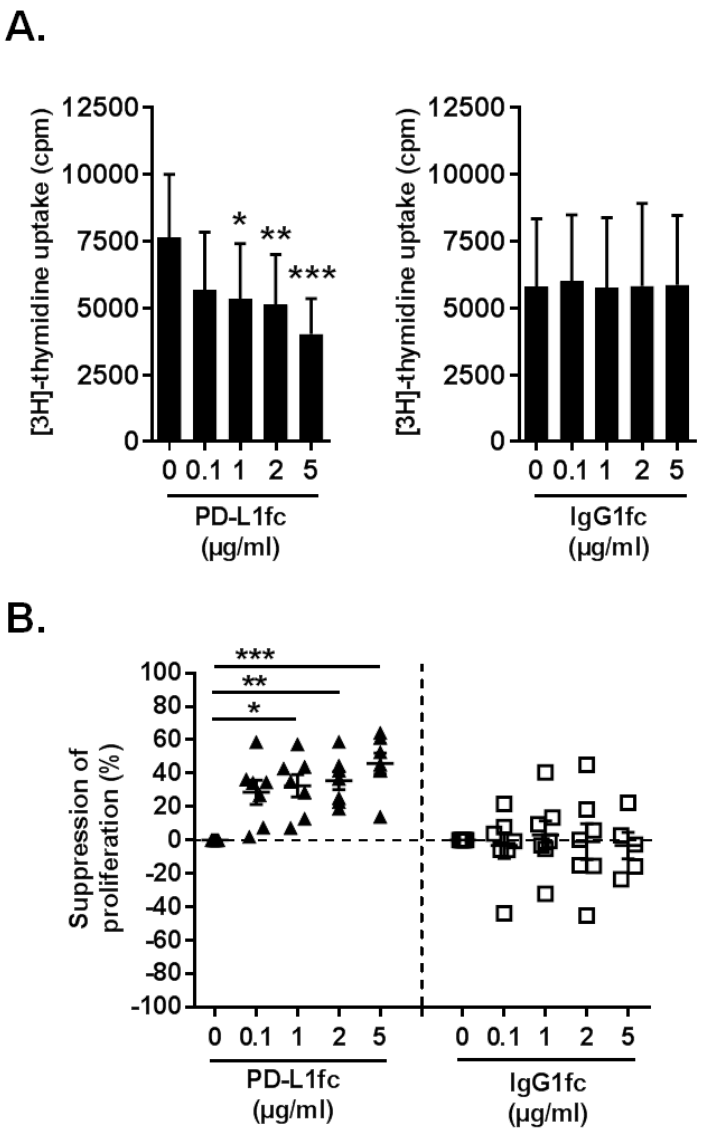

Figure 4.3 PD-1 ligation reduces proliferation of CD4+ T cells from healthy donors.
CD4+ T cells were isolated from HC PBMC and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc/IgG1fc (0, 0.1, 1, 2 and 5 µg/ml). Proliferation was assessed on day 5 by [^{3}H]-thymidine incorporation. (A) HC CD4+ T cell proliferation (cpm) and (B) percentage suppression of proliferation following PD-1 ligation by PD-L1fc (n=7) or IgG1fc (n=5-7). Data in A are shown as the mean ± SEM of n=5-7. Data (A-B) were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.4

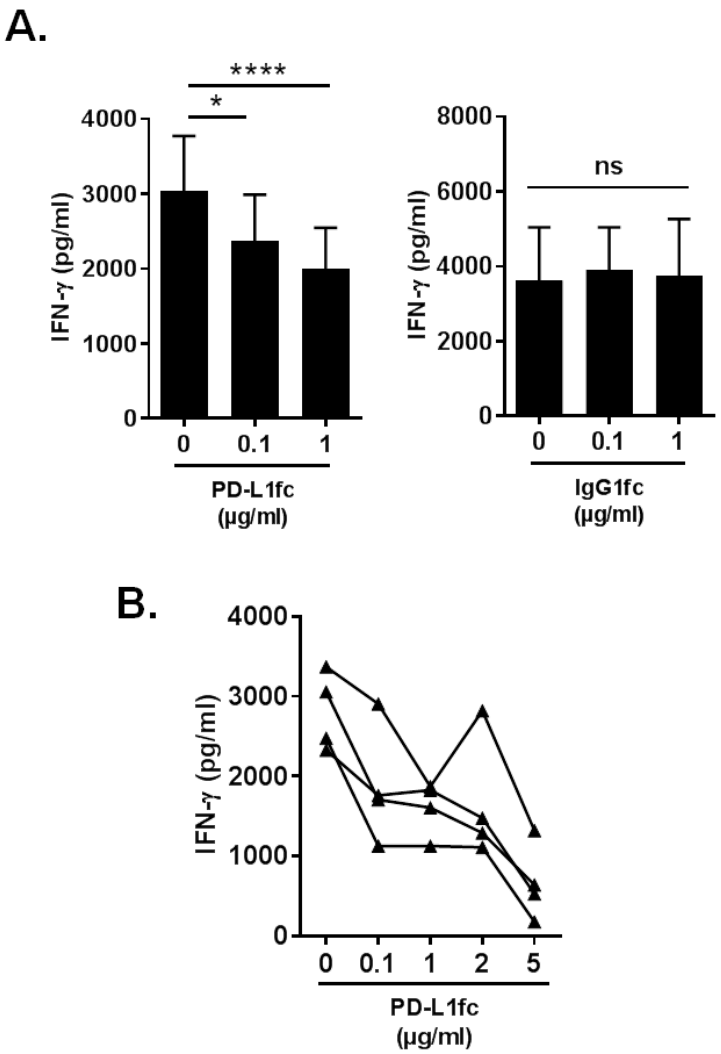

Figure 4.4 PD-1 ligation reduces IFN-γ production by CD4+ T cells from healthy donors. CD4+ T cells from HC PBMC were cultured in plates pre-coated with anti-CD3 mAb (OKT3) and PD-L1fc/IgG1fc. Supernatants were collected at day 5 and tested by ELISA for IFN-γ production. (A) IFN-γ production in HC CD4+ T cell cultures in presence of PD-L1fc (n=11) and IgG1fc (n=5) (0, 0.1 and 1 µg/ml). (B) IFN-γ production in HC CD4+ T cell cultures in presence of PD-L1fc (0, 0.1, 1, 2 and 5 µg/ml; n=4). Data in A are shown as the mean ± SEM. Data (A-B) were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

4.2.2 PD-1 ligation of HC, RA and PsA CD4+ T cells.

The experiments described in the previous section have shown that *in vitro*, PD-1 is functionally capable of modulating both proliferation and cytokine production of healthy human CD4+ T cells. They also provide a reliable experimental system to investigate whether the inflammatory milieu typically found in RA and PsA has a direct effect on PD-1-mediated regulation. It was shown (chapter 3) that significantly increased percentages of PD-1+ cells are found within RA and PsA SF CD4+ T cells (identified either by CD3+CD14-CD4+ cells or CD3+CD8- cells) compared to PB. These data suggest that CD4+ T cells at the site of inflammation seem to be suited with a highly expressed control mechanism to self-regulate proliferation and cytokine production.

To investigate whether PD-1 ligation is functional in patient-derived cells, CD4+ T cells were initially isolated from the peripheral blood of RA patients and cultured in presence of increasing concentrations of PD-L1fc (low range) as previously described. As a control, healthy CD4+ T cells were cultured in parallel and cell proliferation was compared between the two sets of donors. In the absence of PD-L1fc, HC and RA CD4+ T cells from peripheral blood had a similar proliferative capacity and no significant difference was found (Figure 4.5A).

HC and RA CD4+ T cells were then cultured in the presence of increasing concentrations of PD-L1fc. In contrast to healthy CD4+ T cells, RA cells appeared to be resistant to PD-1-mediated suppression of T cell proliferation (Figure 4.5B, 4.5C). Suppression of proliferation of HC PB CD4+ T cells was ~25% and ~35% in presence of 0.1 and 1 μg/ml of PD-L1fc, respectively, compared to medium only (Figure 4.5C). Conversely, suppression of proliferation was largely unchanged for RA

PB CD4+ T cells (~1% and ~5% in presence of 0.1 and 1 μg/ml of PD-L1fc compared to medium only). These data show that peripheral blood RA CD4+ T cells are resistant to PD-1-mediated regulation as compared to healthy cells in the selected experimental settings.

The above experiments were performed comparing CD4+ T cells from the peripheral blood of healthy donors and RA patients. Next, the proliferation and cytokine production of CD4+ T cells from the site of inflammation was tested in the same experimental conditions. To address this, new cohorts of RA and PsA patients with joint effusions were selected and CD4+ T cells isolated from PBMC and paired SFMC were cultured in parallel in absence or presence of PD-L1fc or IgG1fc as previously described. For these sets of experiments, cells were cultured using a broader concentration range of PD-L1fc ligand (0, 0.1, 1, 2 and 5μg/ml).

RA and PsA CD4+ T cell proliferation, in absence of PD-L1fc, was not significantly different between PB and SF (Figure 4.6A). In the presence of increasing doses of PD-1 ligand, RA and PsA PB CD4+ T cells were resistant to PD-1-mediated suppression of proliferation as were the paired synovial fluid CD4+ T cells (Figure 4.6C, 4.6D). Importantly, the IgG1fc control had no effect on CD4+ T cell proliferation (Figure 4.6B).

Interestingly, the fact that T cells from the site of inflammation are highly PD-1 positive, even without *in vitro* TCR stimulation, did not seem to play a role in our experimental settings. Indeed, no decrease in PD-1-mediated suppression of T cell proliferation was found, even when the ligand was used at the highest concentration of 5μg/ml. To further confirm these findings, supernatants from RA and PsA cell cultures were tested for the presence of IFN-γ. In absence of PD-L1fc, significantly higher levels of IFN-γ (~5.4 folds higher) were detected in SF-derived CD4+ T cell

cultures compared to PB (Figure 4.7A). This is in line with the literature and likely reflects the activated status of the cells from the site of inflammation. Importantly and in agreement with proliferation data, IFN-γ levels in the supernatants were not significantly affected by increasing concentration of PD-L1fc (Figure 4.7B).

Although these results did not prove that PD-1 signalling is intrinsically compromised, they demonstrate that RA and PsA CD4+ T cells display resistance to PD-1-mediated regulation under the described experimental conditions. This suggests that the particular proinflammatory environment found in patients with RA or PsA might have a role in influencing how CD4+ T cells are regulated via the PD-1 receptor.

Figure 4.5

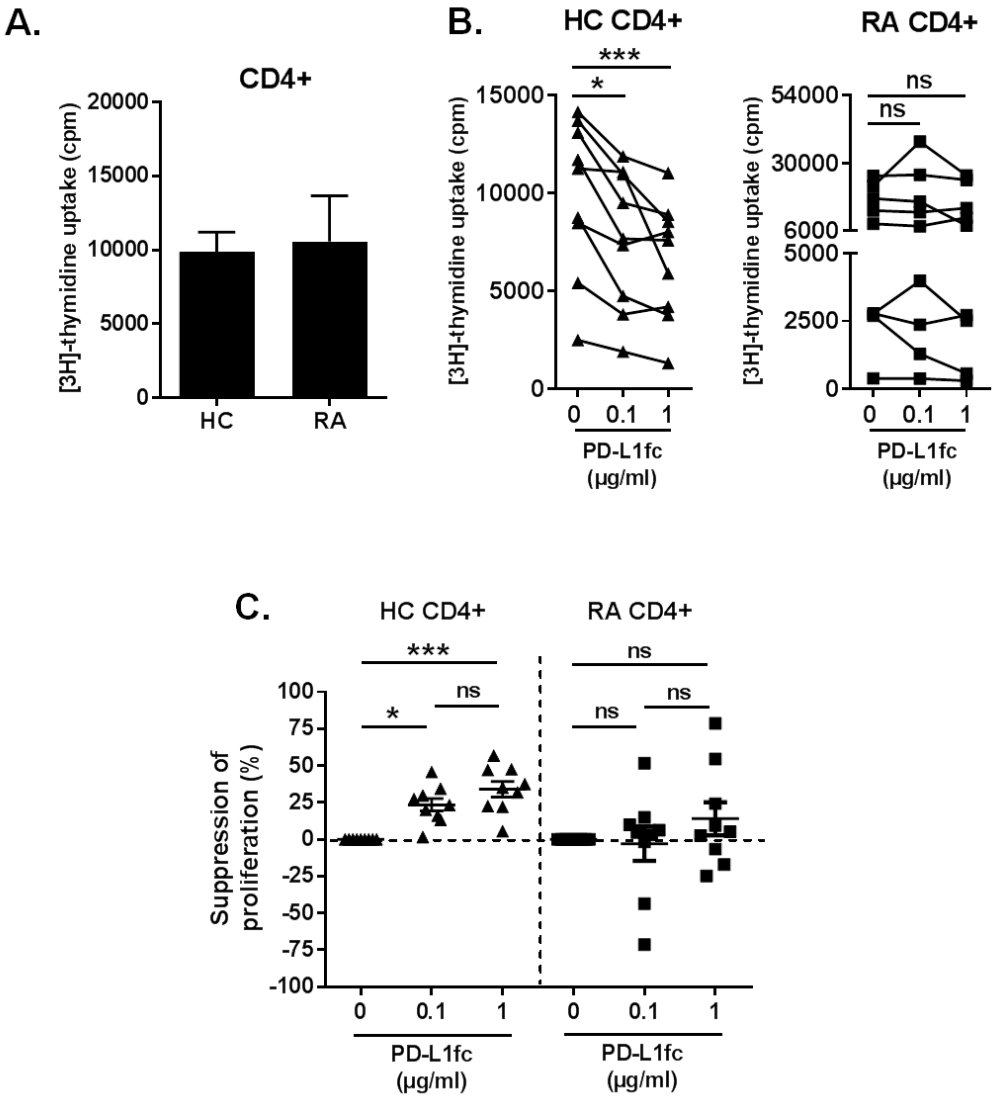

Figure 4.5 PD-1 ligation reduces proliferation of PB CD4+ T cells from healthy donors but not of PB CD4+ T cells from RA patients.
CD4+ T cells were isolated from HC PBMC and RA PBMC (n=9) and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc/IgG1fc (0, 0.1 and 1 µg/ml). (A) HC and RA CD4+ T cell basal proliferation in absence of PD-L1fc (cpm). (B) HC and RA CD4+ T cell proliferation (cpm) and (C) percentage suppression of proliferation following PD-1 ligation by PD-L1fc. Data in (B-C) were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.6

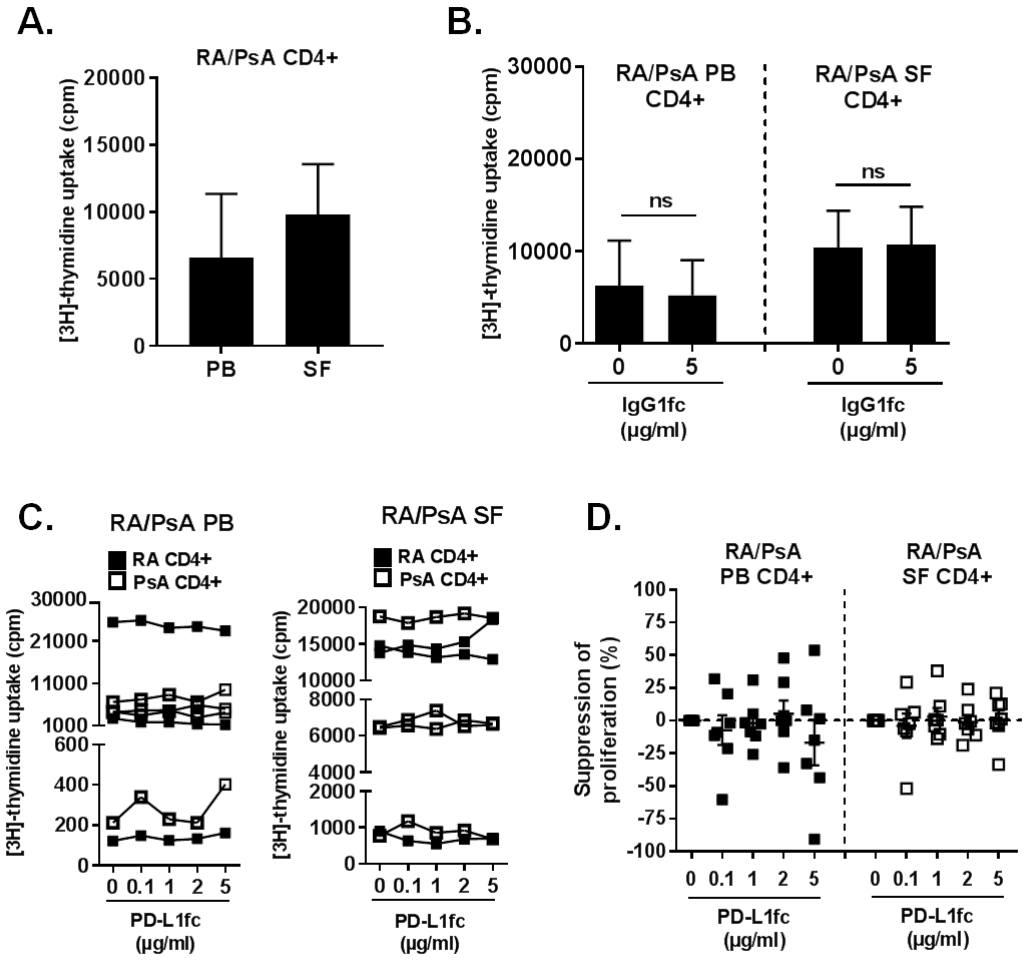

Figure 4.6 PD-1 ligation is unable to reduce proliferation of PB and SF CD4+ T cells from RA and PsA patients.

CD4+ T cells were isolated from RA PBMC and SFMC and PsA PBMC and SFMC (n=3 RA PB/SF; n=4 PsA PB/SF) and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc (0, 0.1, 1, 2 and 5 µg/ml) or IgG1fc (0 and 5 µg/ml). (A) RA and PsA CD4+ T cell proliferation (cpm) in absence of PD-L1fc and (B) in presence of 0 and 5 µg/ml of IgG1fc control. (C) Cell proliferation (cpm) and (D) suppression of proliferation in presence of PD-L1fc. Data were analysed by Wilcoxon test (A), Mann Whitney test (B) and Friedman Test with Dunn's Multiple Comparison test (C and D). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.7

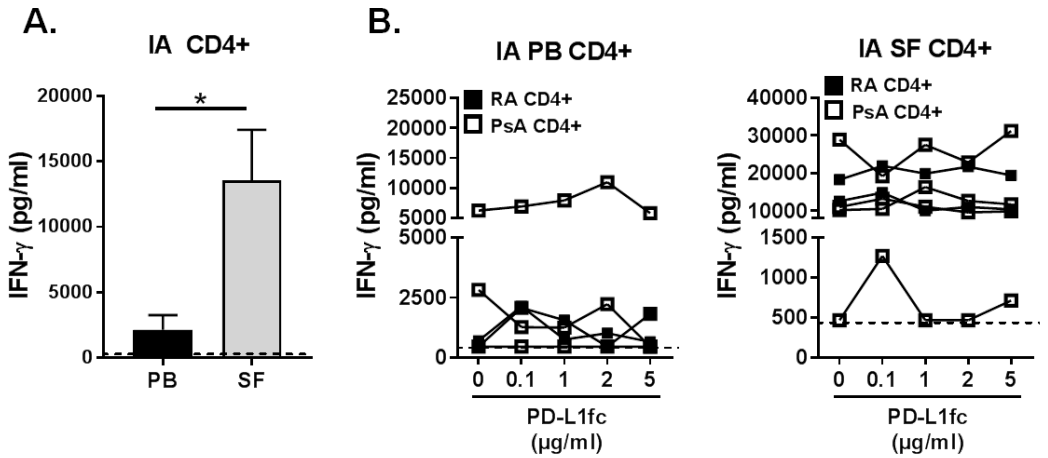

Figure 4.7 PD-1 ligation is unable to reduce IFN-γ production by PB and SF CD4+ T cells from RA and PsA patients.
CD4+ T cells from RA and PsA PBMC and SFMC (n=2 RA PB/SF; n=3-4 PsA PB/SF) were cultured in plates pre-coated with anti-CD3 mAb (OKT3; 1.5µg/ml) and PD-L1fc/IgG1fc. Supernatants were collected at day 5 and tested by ELISA for IFN-γ production. (A) Levels of IFN-γ in paired IA PB and IA SF CD4+ T cell cultures in absence of PD-L1fc. (B) Levels of IFN-γ in IA PB and IA SF CD4+ T cell cultures in presence of increasing concentration of PD-L1fc. Data were analysed by Wilcoxon test (A) and by Friedman Test with Dunn's Multiple Comparison test (B). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Dotted line indicate minimum detection limit.

4.2.3 Levels of proinflammatory cytokines in RA and PsA serum and synovial fluid and in PBMC and SFMC cell supernatants

The data so far demonstrated that CD4+ T cells from PB and SF of patients with RA or PsA are resistant to PD-1 ligation as compared to healthy controls. RA and PsA CD4+ T cells, especially those found in the synovial fluid, derive from a highly proinflammatory environment and they are likely to be exposed to continuous cytokine stimulation.

Next, the presence of proinflammatory cytokines in RA and PsA serum and synovial fluid as well as their ability to interfere with PD-1-mediated T cell suppression was investigated. Previously generated Luminex data available in the laboratory were analysed to determine the levels of the proinflammatory cytokines IL-7, IL-15, TNFα, IL-6 and IL-1β in RA-derived paired sera and SF as compared to healthy serum. In the RA sera, only slight and not significant increases of IL-7 and IL-15 were found when compared to HC (Figure 4.8). Conversely, significantly increased amounts of IL-7 (~200 pg/ml) and IL-15 (~750 pg/ml) were detected in RA SF when compared to RA serum. In this particular set of patients, TNFα and IL-1β were detected at higher levels only in 5/12 RA sera and SF compared to HC sera and these differences did not reach statistical significance. Conversely, IL-6 was increased in the RA SF (~1000 pg/ml) as compared to paired RA serum and HC serum (<50 pg/ml) (Figure 4.8).

To further investigate the inflammatory nature of the RA and PsA arthritic joint, two additional sets of RA and PsA patients were selected. In these new experiments, cytokine levels were determined in the serum and paired SF as compared to healthy serum and serum and SF from disease control patients with osteoarthritis (OA). Figure 4.9 shows that in the PsA sera, TNFα and IL-6 were significantly

increased when compared to HC serum while in the analysed RA sera only IL-6, but not TNFα, was increased. IL-1β was undetected in the selected samples. Conversely, strong and significant increases in all three cytokines were detected in both RA and PsA synovial fluid when compared to HC or OA controls (Figure 4.10A, 4.10B) confirming the inflammatory nature of RA and PsA synovial fluid. Data show that in the selected RA and PsA sets there was a common proinflammatory signature for TNFα, IL-6 and IL-1β as evidenced by similar cytokine levels between the two diseases (Figure 4.10C). The levels of other cytokines including IFN-γ, IL-4, IL-10, IL-22, IL-23, IL-25, IL-31 and IL-33 were also investigated but no statistically significant variations or clear expression patterns were detected when compared to controls and no further investigation was performed (data not shown).

As a further confirmation of these data and to investigate whether cells from the site of inflammation could also produce these cytokines *in vitro*, paired RA- and PsA-derived whole PBMC and SFMC cell cultures were set up. Cells were cultured for 48 hrs in presence of soluble anti-CD3 mAb (OKT3; 100 ng/ml) and day 2 supernatants were analysed by luminex. TNFα, IL-6 and IL-1β were detected in the supernatants of both PBMC and SFMC cultures and cytokine levels were significantly higher in the SFMC cultures compared to PBMC (Figure 4.11). The data presented in this section indicate that TNFα, IL-6 and IL-1β are indeed present in RA and PsA serum and SF and they can be further detected *in vitro* in the supernatants of activated PBMC and SFMC cultures.

Next, the ability of TNFα, IL-6 and IL-1β cytokines to modulate PD-1-mediated suppression of HC CD4+ T cell proliferation *in vitro* was investigated.

Figure 4.8

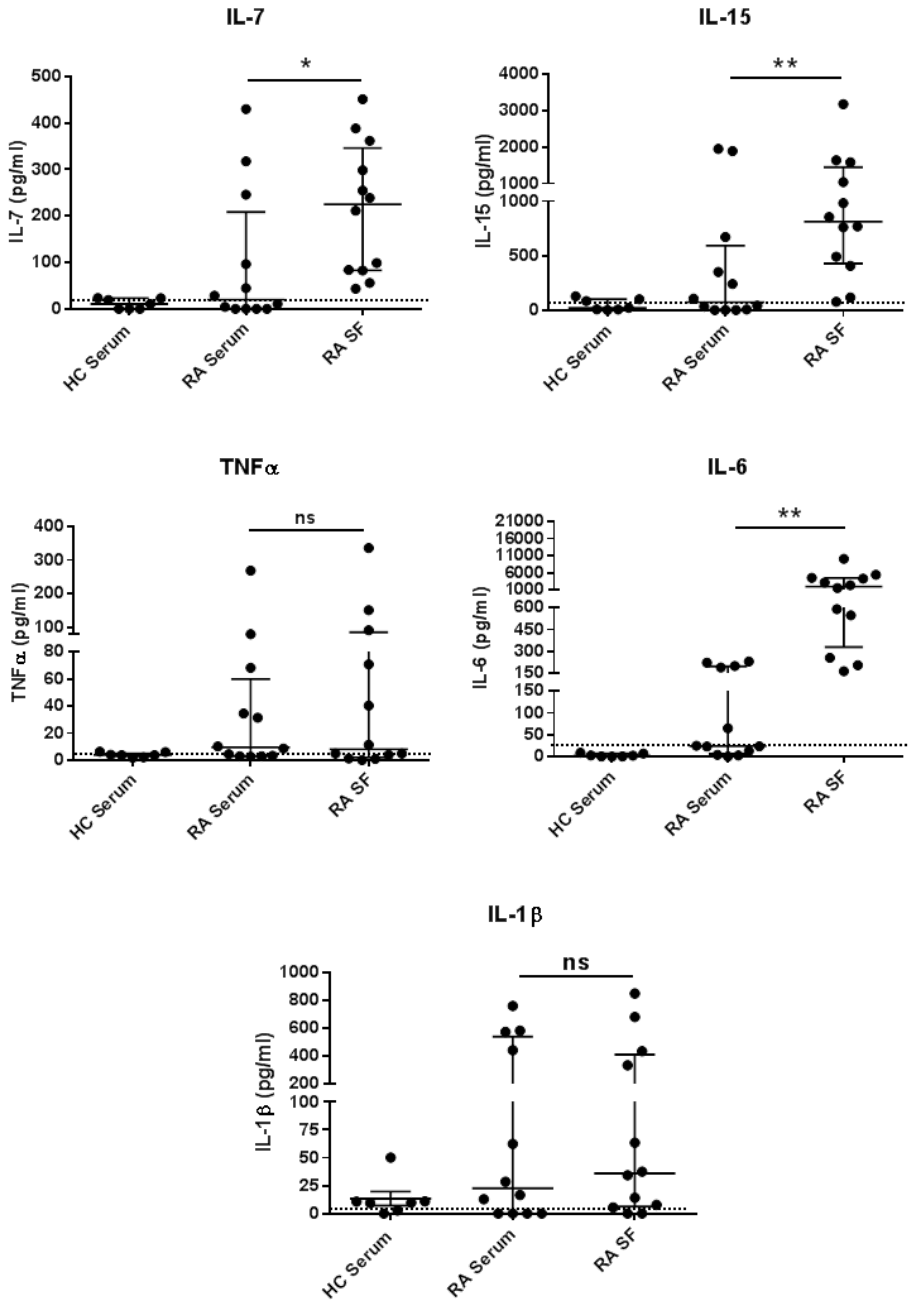

Figure 4.8 Cytokine levels for IL-7, IL-15, TNFα, IL-6 and IL-1β in the serum and SF of RA patients and in the serum of healthy controls (Set I).
Levels of IL-7, IL-15, TNFα, IL-6 and IL-1β in the serum and paired SF of patients with RA (n=12) or in HC serum (n=7) are shown. Data were measured by Luminex and analysed by Wilcoxon matched-pairs signed rank test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Graphs show Median + IQR. Dotted line indicates minimum detection limit.

Figure 4.9

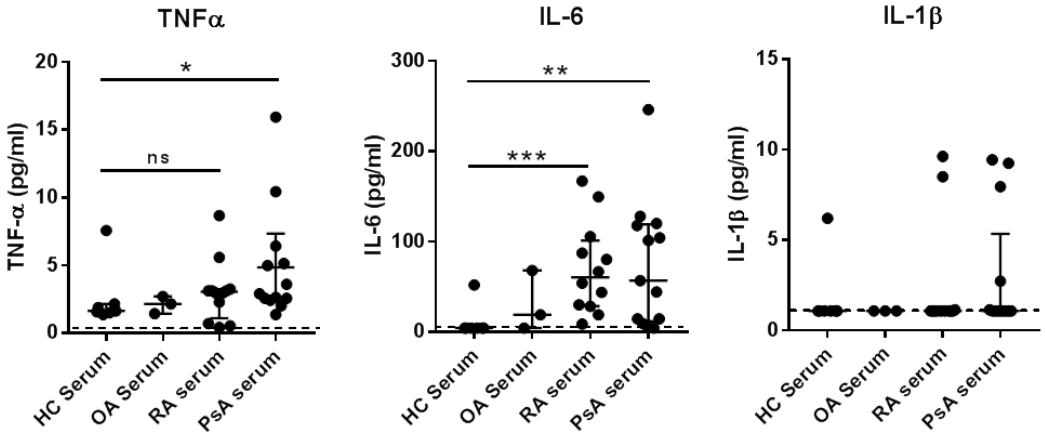

Figure 4.9 Levels of proinflammatory cytokines TNFα, IL-6 and IL-1β in the serum of RA and PsA patients (Set II).
Levels of the proinflammatory cytokines TNFα, IL-6 and IL-1β in the serum of patients with RA (n=12), PsA (n=12), OA (n=3-4) or in HC serum (n=7) are shown. Data were measured by Luminex and analysed by Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Graphs show Median + IQR. Dotted line indicates minimum detection limit.

Figure 4.10

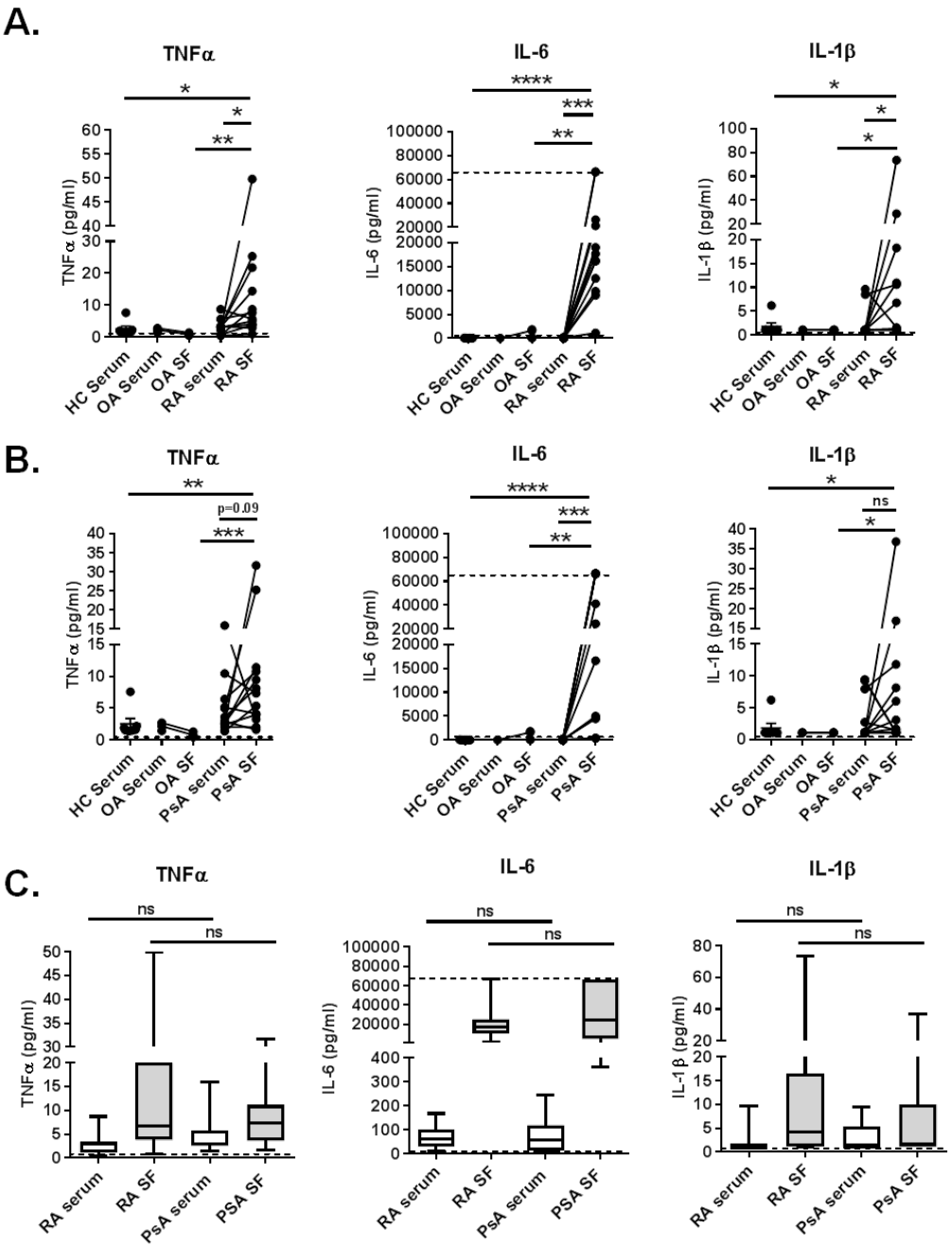

Figure 4.10 Analysis of proinflammatory cytokines TNFα, IL-6 and IL-1β in the serum and SF of RA and PsA patients (Set II).

(A-B) Levels of proinflammatory cytokines TNFα, IL-6 and IL-1β in paired RA and PsA serum/synovial fluid (SF) (n=12), in OA (disease control) serum/SF (n = 3-4) and in HC serum (n=7). (C) Comparison of proinflammatory cytokines TNFα, IL-6 and IL-1β levels between RA and PsA. Data in (A) and (B) were analysed by Wilcoxon matched-pairs signed rank test for RA/PsA Serum vs. RA/PsA SF and Mann-Whitney test for RA/PsA SF Vs HC serum or OA SF. Data in (C) were analysed by Mann-Whitney test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Dotted line indicates minimum/maximum detection limit.

Figure 4.11

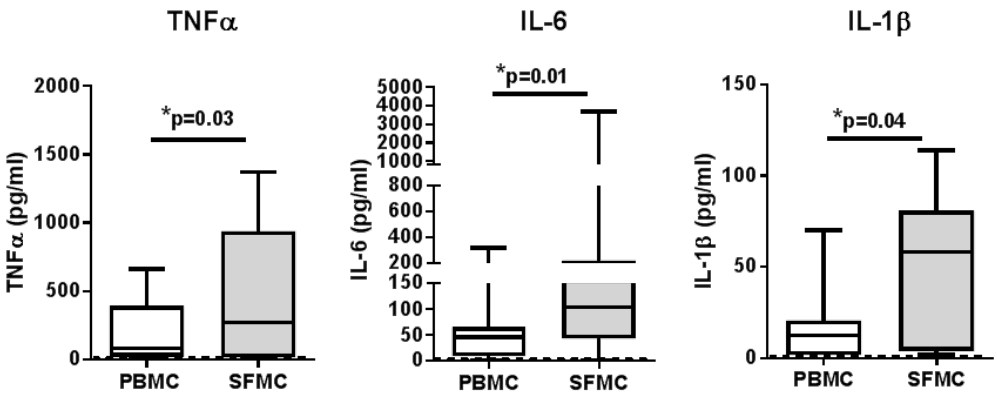

Figure 4.11 Cells from the site of inflammation produce higher levels of TNFα, IL-6 and IL-1β compared to PB-derived cells.
Inflammatory arthritis (RA and PsA) paired PBMC and SFMC were cultured with anti-CD3 mAb (100 ng/ml) for 48 hrs and supernatants were analysed by Luminex (n=8; of which n=6 RA, n=2 PsA). Data were analysed by Wilcoxon matched-pairs signed rank test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Dotted line indicates minimum detection limit.

4.2.4 The effect of proinflammatory cytokines on PD-1-mediated T cell regulation

To elucidate whether inflammation had a functional impact on PD-1-mediated suppression of CD4+ T cell proliferation, IL-15 and IL-2 were initially tested for their ability to negatively modulate PD-1 ligation *in vitro* as previously reported by two different studies (60, 369). Anti-CD3 activated HC CD4+ T cells were cultured in the presence of increasing concentration of PD-L1fc (0, 0.1 and 1μg/ml) and IL-15 or IL-2 (5 ng/ml) were added from the start of the culture.

Figures 4.12A and 4.12B show that in 3 representative HC donors and in a total of n=7 independent experiments, IL-15 consistently abrogated the suppressive effect of PD-1 ligation resulting in increased cell proliferation. In a similar way to IL-15, IL-2 was also able to modulate cell proliferation in presence of the PD-L1fc ligand (Figure 4.12C).

Next, the ability of TNFα, IL-6 or IL-1β to abrogate PD-1-mediated ligation was evaluated. In the first set of experiments, when HC cells were cultured in the presence of each cytokine added singularly but without PD-L1 ligand, no significant change in cell proliferation was observed (Figure 4.13A). HC CD4+ T cells were also cultured with increasing concentrations of PD-L1fc (0, 0.1 and 1 μg/ml) in the absence (medium only) or presence of TNFα, IL-6 or IL-1β (10 ng/ml). As expected, in PD-L1fc + medium-only conditions CD4+ T cell proliferation decreased in a dose-dependent fashion while no change in proliferation was detected in presence of the IgG1fc control (Figure 4.13 B).

Notably, addition of TNFα, IL-6 or IL-1β consistently counteracted PD-1-mediated regulation as shown by the lack of a significant decrease in cell proliferation between the different conditions (Figure 4.13C). These results support the hypothesis

that a pool of inflammatory cytokines found in RA and PsA patients can negatively modulate the suppressive effects of PD-1 ligation on CD4+ T cells.

To assess whether this cytokine-mediated effect could be reversed leading to restored PD-1-mediated regulation, new experiments using neutralising antibodies were performed. To block the biological effect of the cytokines, anti-TNFα (adalimumab), anti-IL-6R (tocilizumab) or anti-IL-1β antibodies were added at the start of the culture together with the respective target cytokine.

To verify that the cytokines and blocking antibodies used in these new sets of experiments were fully functional, supernatants from TNFα and IL-6-stimulated cells cultured in absence or presence of adalimumab and tocilizumab were screened by luminex (for TNFα and IL-6) and by ELISA (for IL-10) and compared to medium only conditions (Figure 4.14). Data in Figure 4.14A show that, as expected, TNFα was significantly increased in the supernatants of TNFα-stimulated cultures while no TNFα was detected in TNFα + adalimumab stimulated cultures. Conversely, IL-10 was decreased following TNFα stimulation but increased in presence of TNFα + adalimumab. This is in line with previous work from our lab (370, 371).

In IL-6-stimulated cultures, IL-6 and IL-10 were both significantly increased compared to medium only while presence of anti-IL6R antibody tocilizumab significantly reduced both cytokines. IL-10 increase following IL-6 stimulation has been previously reported (372). These results validated the biological activity of our cytokines and blocking antibodies.

The new set of experiments confirmed that addition of each individual cytokine significantly abrogated the suppressive effects of PD-1 ligation at both 0.1 and 1 μg/ml of PD-L1 and further demonstrated that adalimumab, tocilizumab and anti-IL-1β mAb were able to completely reverse the cytokine-mediated effects.

Representative data from 1 donor (Figure 4.15A) showing cell proliferation and cumulative data (Figure 4.15B, 4.15C) displaying suppression of proliferation at two different concentrations of PD-L1fc are shown.

Additional experiments were carried out with higher concentrations (2 and 5 μg/ml) of PD-L1fc and IgG1fc control in absence or presence of TNFα or IL-6 and with adalimumab or tocilizumab. In these new sets of experiments TNFα and IL-6 were able to modulate PD-1-mediated suppression of proliferation even in the presence of higher concentrations of PD-L1fc ligand. Data in figure 4.16A show the specificity of PD-L1fc and IgG1fc activity while Figure 4.16B shows a representative experiment and cumulative data from three independent donors.

Together, these data add novel insight to the regulation of the PD-1/PD-L1 pathway showing that T cell and monocyte-derived inflammatory cytokines TNFα, IL-6 and IL-1β, which are typically associated with inflammatory arthritis, have a profound effect on PD-1-mediated suppression of proliferation. Preliminary data suggest that this might also be the case for IL-2 and IL-15. Furthermore, data show that biologic drugs routinely used in the treatment of RA and PsA patients have the ability to abrogate this cytokine-mediated effect *in vitro*.

Figure 4.12

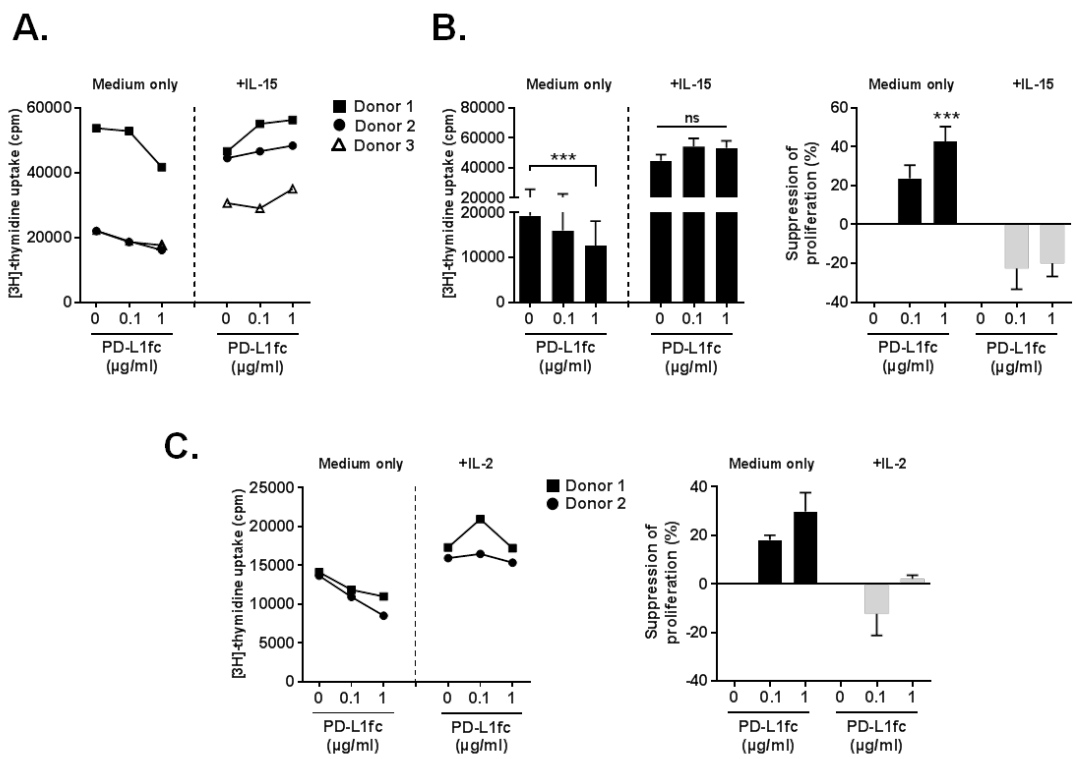

Figure 4.12 IL-15 and IL-2 counteract the PD-L1-mediated suppression of HC CD4+ T cell proliferation.

HC CD4+ T cells were cultured for 5 days in anti-CD3 mAb (OKT3; 1.5 µg/ml) pre-coated plates with increasing concentration of PD-L1Fc (0, 0.1 and 1 µg/ml) and in the absence or presence of either IL-2 or IL-15 (5 ng/ml). (A) Proliferation in n=3 HC donors and (B) cumulative data (n=7) showing HC PB CD4+ T cell proliferation and suppression of proliferation of CD4+ T cells cultured in absence (Medium only) or presence of 5 ng/ml of IL-15. (C) Proliferation and suppression of proliferation of HC PB CD4+ T cells (n=2) cultured in absence (Medium only) or presence of 5 ng/ml of IL-2. Data in (B) are shown as the mean ± SEM of n=7 and were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.13

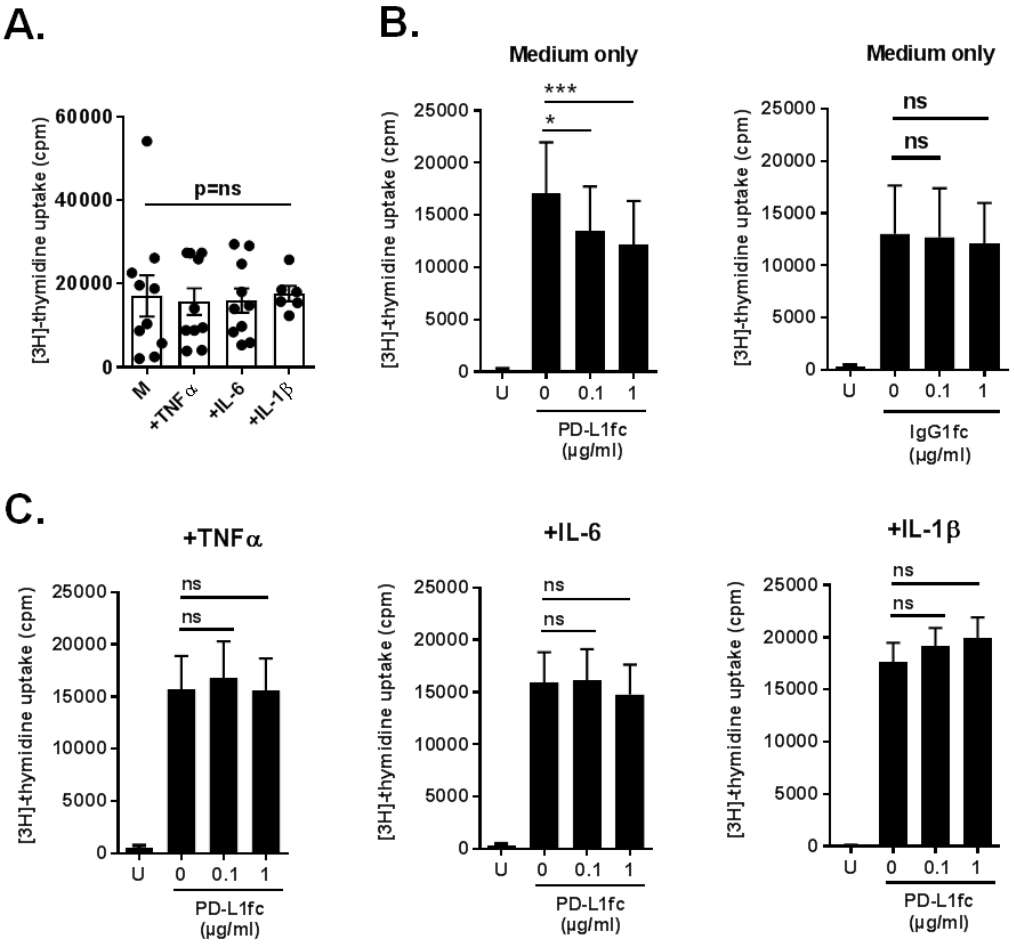

Figure 4.13 TNFα, IL-6 and IL-1β counteract the PD-L1-mediated suppression of HC CD4+ T proliferation.
HC CD4+ T cells were cultured for 5 days in anti-CD3 mAb (OKT3; 1.5 µg/ml) pre-coated plates with increasing concentration of PD-L1Fc (0, 0.1 and 1 µg/ml) and in the absence (Medium only) (n=10) or presence of TNFα (n=10), IL-6 (n=10) or IL-1β (n=6) (10 ng/ml) (A) Proliferation in absence of PD-L1fc and in the presence of specific cytokines. (B) Proliferation in the presence of PD-L1fc or IgG1fc control in absence of specific cytokines. (C) Proliferation in the presence of PD-L1fc and specific cytokines (10ng/ml). Data are shown as the mean ± SEM and were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 4.14

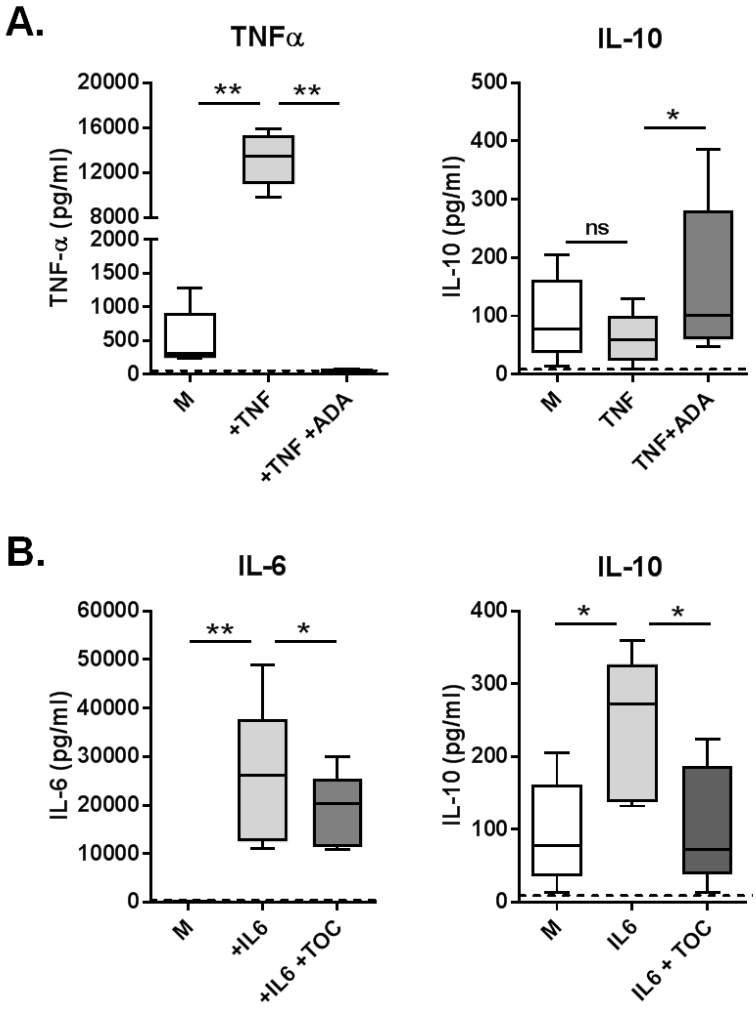

Figure 4.14 Exogenous TNFα and IL-6 are detected in the supernatants and show to have biological activity.
HC CD4+ T cells were cultured for 5 days in anti-CD3 mAb (OKT3; 1.5 µg/ml) pre-coated plates ± TNFα or IL-6 (10 ng/ml) and in the presence of TNFα or IL-6 plus specific blocking antibodies (adalimumab; ADA, tocilizumab; TOC, all at 1 µg/ml) (n=5). At day 5 superatants were collected and analysed by Luminex (TNFα or IL-6) and ELISA (IL-10). Data were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Dotted line indicates minimum detection limit.

Figure 4.15

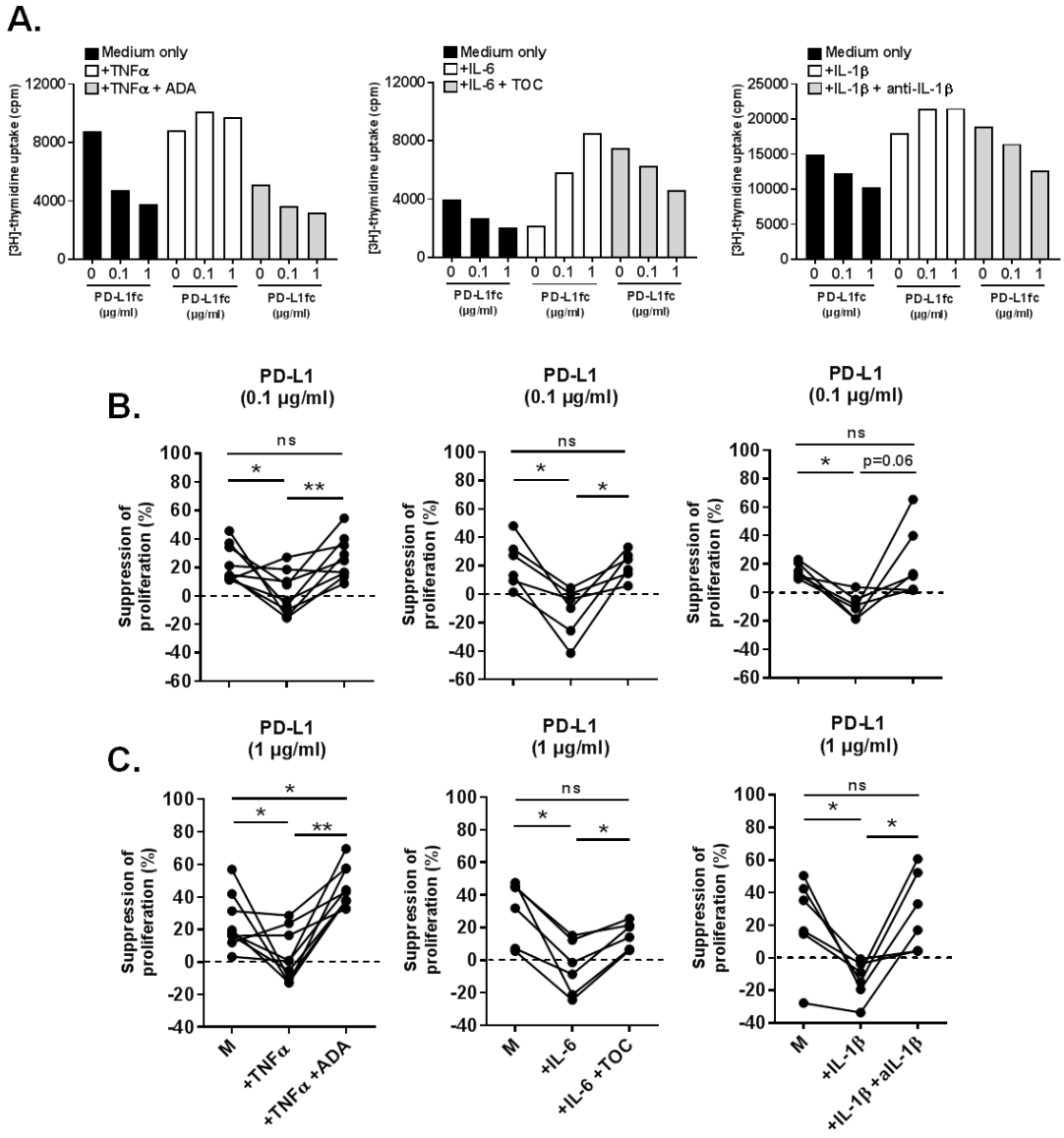

Figure 4.15 TNFα, IL-6 and IL-1β counteract the PD-L1-mediated suppression of proliferation of HC CD4+ T cells and their effect is abrogated by blocking antibodies.
Cell proliferation (cpm) of HC PB CD4+ T cells from one representative experiment (A) and suppression of proliferation (B, C) in CD4+ T cells from HC PB in absence (medium, M) or presence of 10 ng/ml of TNFα (n=9), IL-6 (n=5) or IL-1β (n=6) ± anti-TNFα (adalimumab; ADA), anti-IL-6R (tocilizumab; TOC) and anti-IL-1β (all at 1µg/ml). Black bars: medium only; White bars: + specific cytokine; Gray bars: + specific cytokine and neutralising antibody. Data were analysed by Wilcoxon matched-pairs signed rank test. *$p < 0.05$ and **$p < 0.01$.

Figure 4.16

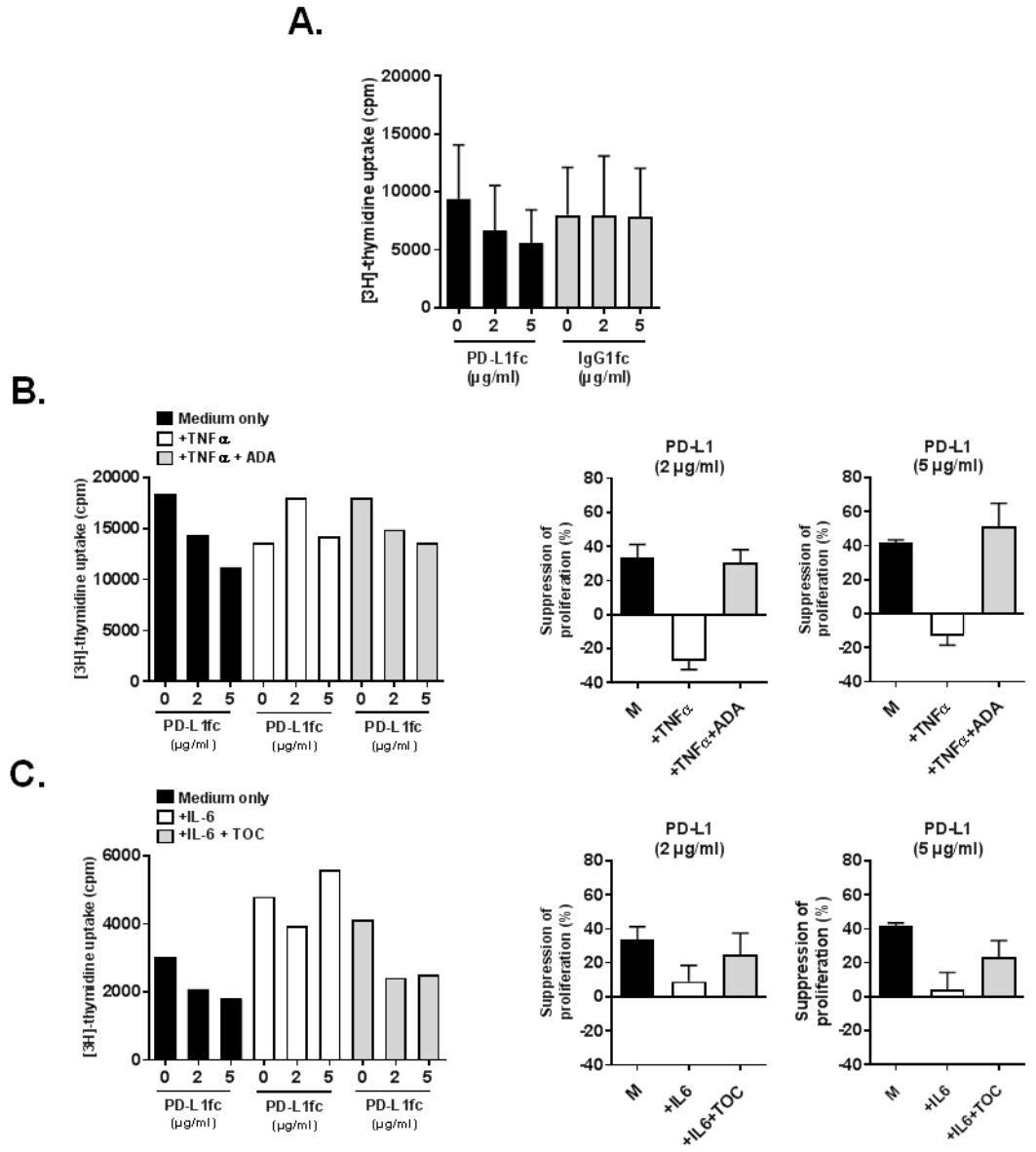

Figure 4.16 TNFα and IL-6 counteract the PD-L1-mediated suppression of HC CD4+ T cell proliferation in presence of 2 and 5 µg/ml of PD-L1fc.
(A) Cell proliferation (cpm) of HC CD4+ T cells in presence of PD-L1fc or IgG1fc control (n=3). (B, C) Representative experiment and cumulative data showing proliferation (n=1) and suppression of proliferation (n=3) in HC CD4+ T cells cultured in absence (medium, M) or presence of 10 ng/ml of TNFα or IL-6 ± anti-TNFα (adalimumab; ADA) or anti-IL-6R (tocilizumab; TOC) (both at 1µg/ml). Data in B and C are shown as the mean ± SEM.

4.2.5 The effect of synovial fluid on PD-1-mediated T cell regulation

Data presented thus far demonstrated that certain proinflammatory cytokines, which are increased in the serum and SF of RA and PsA patients, modulate PD-1-mediated suppression of proliferation of HC CD4+ T cells. It has been reported that addition of autologous synovial fluid to RA PB CD4+ T cell cultures is able to negatively modulate PD-1 ligation (216). However, it is not clear whether this is also the case for PsA synovial fluid and whether PD-1 ligation can be modulated by synovial fluid in HC CD4+ T cells.

To investigate this further, and extend these data to PsA, two synovial fluid samples, 1 RA and 1 PsA, were selected from the RA and PsA sets of patients previously tested for the presence of different proinflammatory cytokines (Figure 4.10). Cytokine levels in the selected RA SF sample were detected as follows: TNFα (5 pg/ml), IL-6 (19 ng/ml) and IL-1β (n.d.) (Figure 4.17A). Cytokine levels in the selected PsA SF sample were detected as follows: TNFα (31.6 pg/ml), IL-6 (24.4 ng/ml) and IL-1β (16.9 pg/ml) (Figure 4.18A). This confirmed the proinflammatory nature of the selected samples.

HC CD4+ T cells from healthy donors were cultured in anti-CD3 mAb (OKT3) coated plates in presence or absence of PD-L1fc and with or without 0.5% sterile acellular SF. In some conditions a combination of 0.5% SF + adalimumab (1 μg/ml) was added from the start of the culture.

Addition of 0.5% RA or PsA synovial fluid to HC CD4+ T cells had two effects. First, it reduced the overall proliferation of HC CD4+ T cells. Second, it consistently reduced PD-1-mediated suppression of proliferation as compared to medium only (Figure 4.17B and 4.18B). TNFα neutralising antibody (adalimumab; 1

μg/ml) added together with RA or PsA synovial fluid from the start of the culture was able to partially rescue the observed SF-mediated effect.

These data, although still preliminary, suggest that inflammatory mediators found in RA and PsA synovial fluid might have the potential to negatively modulate an otherwise functional PD-1 ligation in HC CD4+ T cells and are in line with the proinflammatory cytokine experiments performed in section 4.2.4 as well as with previously reported findings focusing only on RA (216).

Figure 4.17

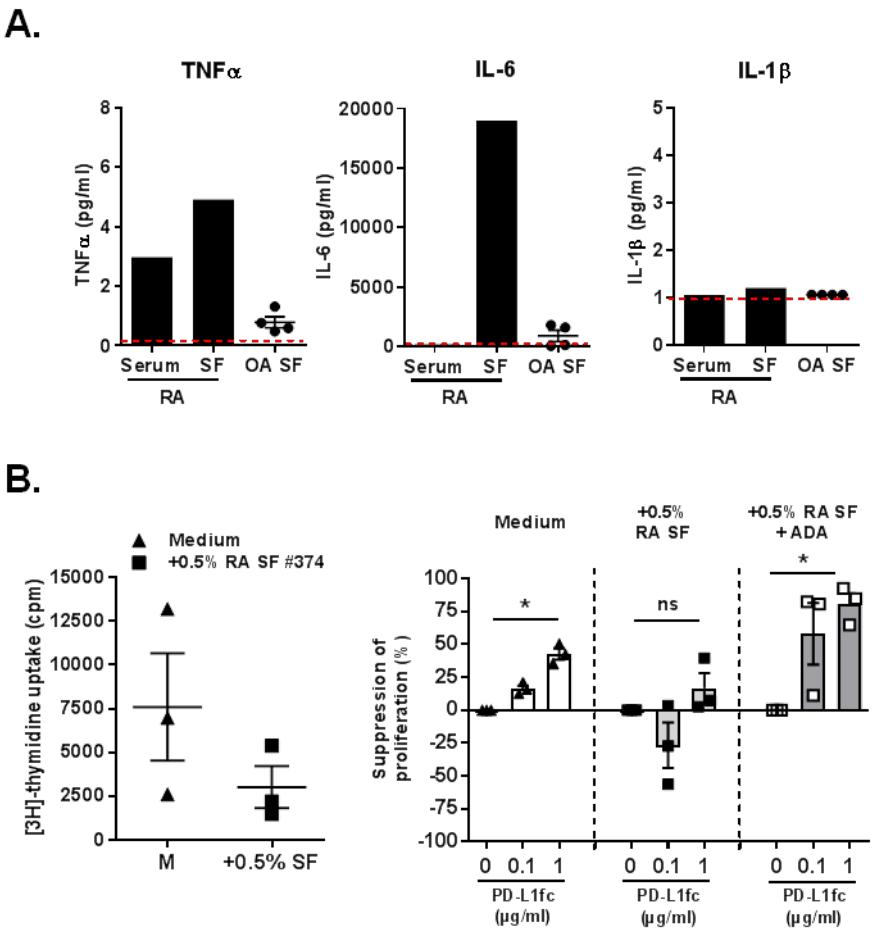

Figure 4.17 RA-derived SF modulates PD-L1-mediated suppression of HC CD4+ T cell proliferation.
HC CD4+ T cells were cultured for 5 days in anti-CD3 mAb (OKT3; 1.5 µg/ml) pre-coated plates with increasing concentration of PD-L1Fc (0, 0.1 and 1 µg/ml). (A) Levels of TNFα, IL-6 and IL-1β were determined by Luminex in the selected RA SF and in 4 OA SF samples. (B) Proliferation (cpm) in absence or presence of 0.5% of RA SF and PD-L1-mediated suppression of proliferation in HC PB CD4+ T cells (n=3) in absence (Medium) or presence of 0.5% of the same RA SF ± anti-TNFα (adalimumab; ADA). Data were analysed by Friedman Test with Dunn's Multiple Comparison test. *p < 0.05, **p < 0.01 and ***p < 0.001. Dotted line indicates minimum detection limit.

Figure 4.18

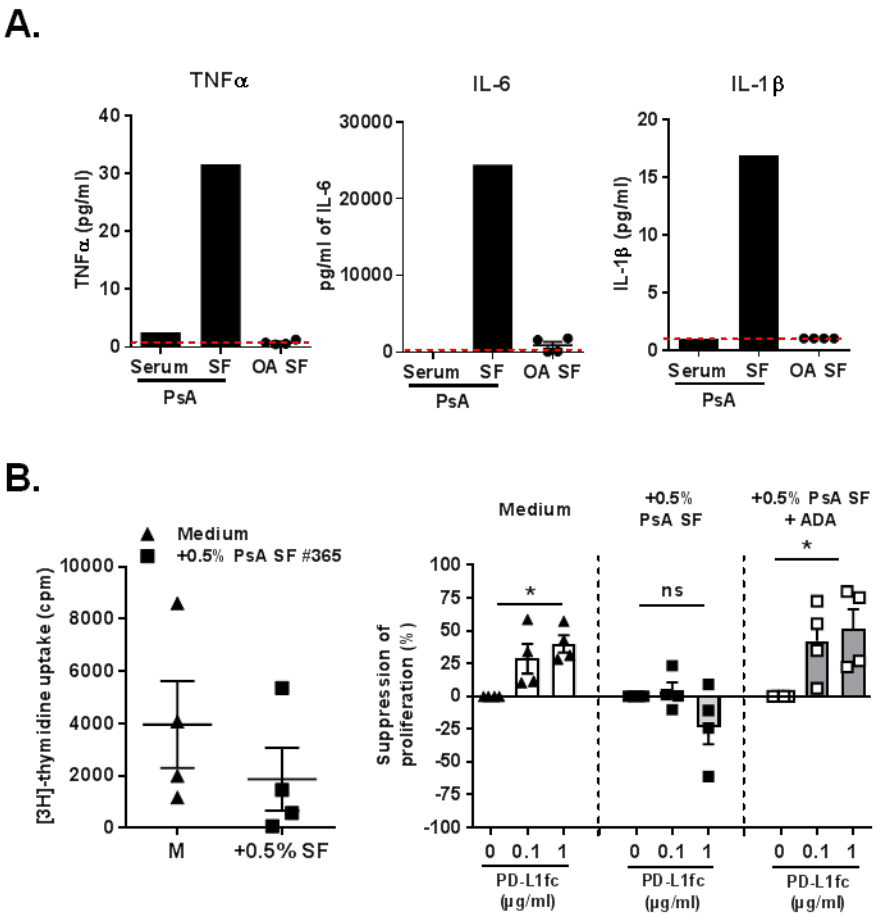

Figure 4.18 PsA-derived SF modulates PD-L1-mediated suppression of HC CD4+ T cell proliferation.
HC CD4+ T cells were cultured for 5 days in anti-CD3 mAb (OKT3; 1.5 µg/ml) pre-coated plates with increasing concentration of PD-L1Fc (0, 0.1 and 1 µg/ml). (A) Levels of TNFα, IL-6 and IL-1β were determined by Luminex in the selected PsA SF and in 4 OA SF samples. (B) Proliferation (cpm) in absence or presence of 0.5% of PsA SF and PD-L1-mediated suppression of proliferation in HC PB CD4+ T cells (n=4) in absence (Medium) or presence of 0.5% of the same PsA SF ± anti-TNFα (adalimumab; ADA). Data were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Dotted line indicates minimum detection limit.

4.3 Discussion

The data presented in this chapter provide new findings regarding the ability of the PD-1 receptor to regulate cell proliferation in human CD4+ T cells. Firstly, CD4+ T cells were stimulated via the TCR in presence or absence of a PD-L1fc chimera to mimic PD-1 ligation. This assay demonstrated that CD4+ T cells from the PB of patients with RA or PsA are resistant to PD-1-mediated suppression of proliferation as compared to healthy CD4+ T cells. Next, it was established that SF-derived CD4+ T cells from either RA or PsA patients are also resistant to PD-1-mediated regulation despite being highly PD-1 positive. Finally, it was shown that the levels of proinflammatory cytokines TNFα, IL-6 and IL-1β are increased in the synovial fluid of both RA and PsA patients compared to healthy and osteoarthritis (OA) controls and that these cytokines are negative modulators of PD-1 function *in vitro*. This in line with what is observed with cytokines IL-2 and IL-15. Importantly, biologics adalimumab (anti-TNFα) and tocilizumab (anti-IL-6R) as wells as anti-IL-1β blocking antibody, were found to significantly counteract these cytokine-mediated effects. Preliminary data also suggest that RA or PsA synovial fluid added to HC CD4+ T cell cultures negatively modulate PD-1-mediated suppression of proliferation.

Several studies have reported the ability of PD-1 to regulate cell proliferation and cytokine production in healthy T cells. First described by Ishida *et al.* (56) and Agata *et al.* (93) in mice and further characterised by Freeman *et al.* (60) in both mice and human, the PD-1 receptor has emerged in more recent years as a key regulator for T cell activation (26, 61, 102, 369). Freeman *et al.* (60) have shown that TCR activation of splenic murine T cells and human CD4+ T cells, in presence of PD-L1, results in inhibition of cell proliferation and cytokine production *in vitro* and that

the outcome of the interaction between PD-1 and PD-L1 in human CD4+ T cells depends on the strength of TCR and CD28 signals. The same group has shown that this is also the case in presence of PD-1 second ligand PD-L2 (61).

The importance of PD-1 engagement with its ligand PD-L1 has been extensively characterised in murine models of autoimmune disease. Wang *et al.* investigated the role of PD-L1 in autoimmune diabetes by transgenically overexpressing PD-L1 in pancreatic β-cells in non-obese diabetic (NOD) mice and described decreased insulitis severity and delayed disease onset compared to controls (373). PD-1 activation via PD-L1 has also been shown to inhibit T cell-mediated immune responses and prolong allograft survival in multiple experimental transplantation models (374, 375). In murine experimental models of RA, it was shown that enhancement of PD-1 crosslink via intraperitoneal injection of soluble murine PD-L1fc fusion protein resulted in less severe arthritis and reduced T cell proliferation supporting a role for PD-1 in the regulation of inflammation (216). Similarly, another study showed that administration of PD-L1fc to collagen-induced arthritis (CIA) mice reduces clinical arthritis score and serum levels of proinflammatory cytokines IL-17 and IL-23 (215).

The association between polymorphisms in the *PDCD1* human gene and susceptibility to RA, AS and SLE (section 4.1) strongly suggests a role for PD-1 in the progression of different human inflammatory diseases.

Using a validated PD-1 ligation assay (60, 127, 368), this study confirmed that *in vitro*, PD-1 engagement with its ligand PD-L1 leads to a significant and dose-dependent reduction of proliferation and IFN-γ production in healthy control PB-derived CD4+ T cell. This is in agreement with previous work performed on human PB CD4+ T cells (60). Data presented in this chapter also provide novel evidence that

CD4+ T cells from the peripheral blood of RA patients are more resistant to PD-1-mediated suppression of proliferation as compared to HC CD4+ T cells. Interestingly, Raptopoulou *et al.* reported that PD-1 is capable of suppressing proliferation in PB-derived CD4+ T cells from RA patients, OA patients and healthy donors and that only SF-derived RA CD4+ T cells are resistant to PD-1 ligation in presence of low doses of PD-L1 ligand (216). The observed discrepancies might be a consequence of the different cohorts of patients used for such experiments and the different sensitivity of the methods used to detect T cell proliferation between this thesis ([^{3}H]-thymidine assay) and the specific experiment in the cited manuscript which used a carboxyfluorescein succnimidyl ester (CFSE) assay for that specific analysis.

Further indication to support the hypothesis of an impaired PD-1 pathway during chronic inflammation can be found in juvenile idiopathic arthritis (JIA). A study from Wehrens *et al.* showed that CD4+ T cells from the synovial fluid of JIA patients have increased phosphorylation of the kinase PKB (Akt) and are resistant to Treg-mediated suppression. The study also found that TNFα and IL-6, which are present at the site of inflammation, induce Akt activation in CD4+ T cells resulting in resistance to TGFβ and Treg-mediated suppression (376). This is of interest because it has been demonstrated that PD-1 engagement with its ligands leads to inhibition of PI3K activation and reduced Akt phosphorylation (36, 68). Hence, in JIA, PD-1-mediated regulation of SF CD4+T cells might be compromised leading to hyperactivation of Akt.

Importantly, in new sets of experiments performed in this thesis, CD4+ T cells from the blood and paired synovial fluid of RA and PsA patients were found to be equally resistant to PD-1 ligation in terms of suppression of T cell proliferation and IFN-γ production when compared to healthy cells. Results from this thesis further

extended the work from Raptopoulou *et al.*, which focused only on RA (216), and provided novel insight in CD4+ T cell regulation in PsA demonstrating that CD4+ T cells from two different inflammatory arthrides display similar resistance to PD-1-mediated regulation. These findings are interesting as they indicate that the inflammatory milieu associated with chronic inflammation might have a role in modulating PD-1-mediated regulation in T cells.

Different cytokines have been shown to modulate PD-1 function. Bennett *et al.* showed that *in vitro* IL-2, IL-7 and IL-15, but not IL-4 or IL-21, can interfere with PD-1 ligation in HC CD4+ T cells resulting in higher cell proliferation (368). The authors demonstrated that IL-2 induces STAT5 phosphorylation in TCR activated T cells and speculated that because neither IL-4 nor IL-21 can induce pSTAT5, nor can rescue PD-1-mediated inhibition, only those cytokines that activate STAT5 can negatively modulate the PD-1 pathway. Notably, IL-2, IL-7 and IL-15, can all be detected in the circulation and at the site of inflammation of RA and PsA patients (270, 377-379) supporting the observation that different cytokines may interfere with PD-1 mediated suppression of proliferation *in vivo*.

Data analysis performed in this chapter confirmed that increased levels of IL-7 and IL-15 are found in RA SF when compared to RA and HC serum while functional experiments confirmed that IL-15 and IL-2 are able to interfere with PD-1 crosslink *in vitro* as reported by other authors (368, 369). Data from this chapter also show that RA and PsA have a very similar proinflammatory cytokine signature for TNFα, IL-6 and IL-1β and that these cytokines, and possibly acellular SF, are able to reduce PD-1-mediated suppression of proliferation in HC CD4+ T cells. Furthermore, neutralising

antibodies against TNFα, IL-6R and IL-1β can reverse the observed cytokine-mediated effect (380).

Although these results are novel and interesting they do have limitations. Co-stimulation via CD28 (60, 368) and IL-2 production following CD28 engagement (369, 381) have been shown to negatively modulate PD-1 interaction with PD-L1fc or anti-PD-1 antibody leading to increased cell proliferation. Hence, further experiments performed in the presence of soluble anti-CD28 might reveal whether the cytokine-mediated effect observed in TCR-only stimulated cells is still present in presence of co-stimulation. Similarly, further experiments using PD-L2 (61), the second ligand for PD-1, might be useful to further confirm that RA- and PsA-derived T cell, as well as cytokine-stimulated HC T cells, are resistant to PD-1 ligation *in vitro* (see chapter 6, section 6.2.1). Indeed, it cannot be ruled out that the observed effects described in this chapter might be strictly dependent on the selected experimental settings.

The reduced PD-1-mediated regulation observed and described in RA and PsA CD4+ T cells and in healthy cells cultured with proinflammatory mediators raises the question about potential implications in human inflammatory disease. The increased frequencies of PD-1+CD4+ T cells in RA and PsA synovial fluid suggests that, at the site of inflammation, PD-1 might have a role in regulating T cell effectors and not simply act as a marker for cell exhaustion (382, 383). A confirmation of this theory was recently brought by Rao *et al.* (349), which described that following therapy, PD-1^{hi}CXCR5-CD4+ T cells (T_{PH}) were reduced in the blood of RA patients and that such reduction significantly correlated with a lower disease activity. Synovial and blood-derived PD-1^{hi}CXCR5- cells were found to express inflammatory chemokine receptors CCR2, CX3CR1, and CCR5, which are known to mediate migration to the sites of peripheral inflammation (384, 385). Finally, PD-1^{hi}CXCR5- cells had the

ability to produce cytokines IL-21 and CXCL13 upon *in vitro* stimulation and to induce differentiation of memory B cells into plasma cells indicating that these cells are not simply exhausted but instead hold a possible B cell helper function (349).

It is therefore important to further investigate why, during chronic inflammation and despite the presence of high frequencies of PD-1+ T cells in the RA and PsA inflamed joint, the PD-1 pathway seems unable to regulate immune responses. The next chapter explores a possible mechanism by which proinflammatory cytokines modulate PD-1:PD-L1 engagement.

5 Soluble PD-1 (sPD-1) is induced by inflammation and abrogates PD-1:PD-L1 interaction *in vitro*

5.1 Introduction

A large number of soluble proteins corresponding to the extracellular domain of transmembrane receptors and adhesion molecules have been identified and characterised in biological fluids (386). These soluble molecules are able to maintain binding ability and can function as antagonist, agonists, carrier molecules and chaperones modulating immune-regulatory responses and contributing, in some cases, to disease pathology (386, 387). There are two known mechanisms by which soluble receptors are generated. The first mechanism involves proteolytic cleavage of the membrane-bound receptor from the cell surface, which is released in the extracellular milieu (388). Examples of soluble receptors generated via proteolytic cleavage, also known as "shedding", include IL-1R, IL-2R, TNF-R, platelet-derived growth factor (PDGF) and the adhesion molecule L-selectin (CD62L) receptors (387). The second mechanism consists in alternative mRNA splicing which give rise to a polypeptide lacking the transmbrane region (389, 390). This truncated form of the receptor is generally secreted from the cell (387, 391, 392). A few examples of soluble receptors generated through differential mRNA splicing include IL-4R, IL-6R, IL-7R, epidermal growth factor (EGF) receptor and leukemia inhibitory factor (LIF) receptor (387, 391). Recently, Nielsen *et al.* have identified a splice variant of the inhibitory receptor PD-1 which lacks the trans-membrane domain and whose putative translational product is a soluble form of PD-1 (sPD-1) (63). The membrane-bound full length PD-1 isoform (flPD-1) is encoded by 5 exons: exon 1 (leader peptide), exon 2 (extracellular IgV-like domain), exon 3 (transmembrane domain) and exons 4

and 5 (intracellular domain) (393). Four alternatively spliced PD-1 mRNA transcripts in addition to flPD-1 have been identified. These splice variants are PD-1Δex2, PD-1Δex3, PD-1Δex2,3, PD-1Δex2,3,4. All five PD-1 transcripts are constitutively expressed by non-stimulated PBMC and further increased following TCR activation (63). Interestingly, parallel increases in the expression of PD-1Δex3 and flPD-1 were described upon activation. This suggests an important interplay between the two variants and a possible functional antagonism on membrane-bound PD-1 mediated by sPD-1 (63). As discussed in chapter 4, RA and PsA-derived CD4+ T cells are resistant to PD-1-mediated suppression of proliferation. Similarly, HC CD4+ T cells cultured in presence of inflammatory mediators such as TNFα, IL-6 or IL-1β and RA or PsA synovial fluid are also resistant to PD-1-mediated regulation. It was therefore of interest to investigate the mechanism underlying the impaired PD-1-mediated suppression of proliferation with a specific focus on the role of sPD-1.

The aims of this chapter were:

1. To investigate whether proinflammatory cytokines TNFα, IL-6 and IL-1β can induce production of sPD-1 by HC CD4+ T cells.
2. To verify that sPD-1 can be detected *ex vivo* in the serum and SF of inflammatory arthritis patients.
3. To test whether sPD-1 is able to modulate PD-1:PD-L1 interaction in presence of CD4+ T cells only, in CD4+ and CD14+ co-cultures and in a Treg-mediated suppression assay.

5.2 Results

5.2.1 Soluble PD-1 is induced *in vitro* by TNFα and IL-6 in HC CD4+ T cell cultures

The first aim of this chapter was to identify a mechanism to explain, at least in part, why PD-1-mediated suppression of proliferation of HC CD4+ T cells is abrogated in presence of proinflammatory mediators.

For this purpose, TCR-activated HC CD4+ T cells were cultured in the presence of increasing concentrations of PD-L1 (0, 0.1 and 1 μg/ml) and cell supernatants were collected at day 5 and analysed by ELISA for the presence of soluble PD-1 (sPD-1). In some experiments proinflammatory cytokines TNFα, IL-6 or IL-1β (10 ng/ml) used either alone or with specific neutralising antibodies, were added at the start of the culture. In absence of stimulation with exogenous proinflammatory cytokines (medium only conditions), sPD-1 was detected barely above the minimum detection limit and sPD-1 levels were unaffected by the concentration of PD-L1fc (0, 0.1 and 1 μg/ml) (Figure 5.1). Conversely, sPD-1 was increased upon TNFα or IL-6 stimulation (Figure 5.1A), but not in presence of IL-1β stimulation (Figure 5.1B). This effect was observed across all the PD-L1fc concentrations tested and was abrogated in the presence of specific neutralising antibodies adalimumab (anti-TNFα) and tocilizumab (anti-IL-6R) (Figure 5.1A). A representative plot showing the TNFα-, IL-6- and IL-1β-mediated effect in HC CD4+ T cells cultured with 1 μg/ml of PD-L1fc is shown (Figure 5.1C).

To investigate whether TNFα and IL-6-mediated increase in sPD-1 was associated with increased expression of the PD-1Δex3 splice variant, quantitative polymerase chain reaction (qPCR) analysis was performed on HC CD4+ T cells from the TNFα and IL-6 stimulated cultures (Fig. 5.2). Gel electrophoresis was used to

analyse the specificity of the PD-1Δex3 forward and reverse primers (primers sequences can be found in chapter 2). The qPCR product was run on an a 2% agarose gel and the specificity of the PD-1Δex3 forward and reverse primers was confirmed with the identification of a single band of 89 base pairs (bp) size which was the expected length for the PD-1Δex3 transcript. Primers specificity was further confirmed by the presence of single peaks in the specific melting curves of a representative run (Figure 5.2A). In line with the ELISA data, TNFα or IL-6 stimulation consistently increased the PD-1Δex3 transcript as compared to medium only (~50% increase). Importantly, adalimumab or tocilizumab were able to abrogate the cytokine-mediated increase of PD-1Δex3 transcript as previously shown for sPD-1 (Figure 5.2B).

To further test whether cells derived from a proinflammatory milieu are able to produce sPD-1, HC PBMC and paired PBMC and SFMC from patients with RA or PsA were cultured following anti-CD3 mAb stimulation (OKT3; 100ng/ml) for 48 hours and cell supernatants were collected and analysed by ELISA for the presence of sPD-1. Soluble PD-1 was barely detectable in supernatants from HC and RA and PsA PBMC cultures but it was consistently increased in SFMC cultures (Figure 5.3). This indicates that sPD-1 production might be directly consequent to cell exposure to proinflammatory cytokines typically found in the inflamed joint. The results presented in this section indicate that *in vitro* TNFα and IL-6, but not IL-1β, are able to induce sPD-1 in HC CD4+ T cell cultures and that this effect is coupled with increased expression of the PD-1Δex3 splice variant transcript.

The results also indicate that sPD-1 and PD-1Δex3 levels are modulated by adalimumab and tocilizumab and that sPD-1 can be found in patient-derived SFMC culture supernatants.

Figure 5.1

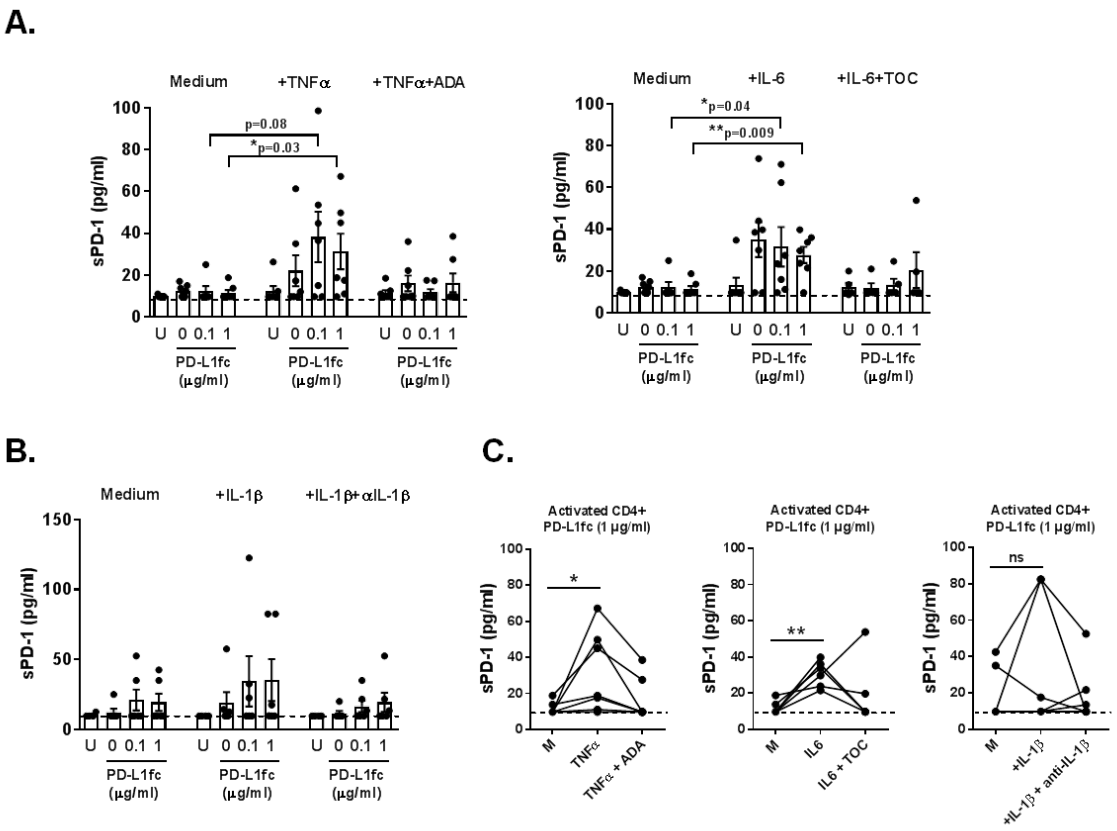

Figure 5.1 Soluble PD-1 (sPD-1) is induced *in vitro* by TNFα and IL-6 in HC CD4+ T cell cultures and decreases in presence of adalimumab and tocilizumab.
(A-C) CD4+ T cells were isolated from HC PBMC and cultured for 5 days in plates pre-coated with anti-CD3 mAb (OKT3; 1.5 µg/ml) and PD-L1fc (0,0.1 1 µg/ml). Supernatants were collected at day 5 and analysed by ELISA. (A-B) HC CD4+ T cells cultured in presence of increasing concentrations of PD-L1fc and stimulated with TNFα (10 ng/ml) or TNFα + adalimumab (ADA; 1 µg/ml) (n=7), IL-6 (10 ng/ml) or IL-6+Tocilizumab (TOC; 1 µg/ml) (n=5-7) and IL-1β (10 ng/ml) or IL-1β+anti-IL-1β (anti-IL-1β; 1 µg/ml) (n=5). (C) Cumulative experiment showing HC CD4+ T cells stimulated in the presence of 1 µg/ml of PD-L1fc. Data in were analysed by Mann-Whitney test. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5.2

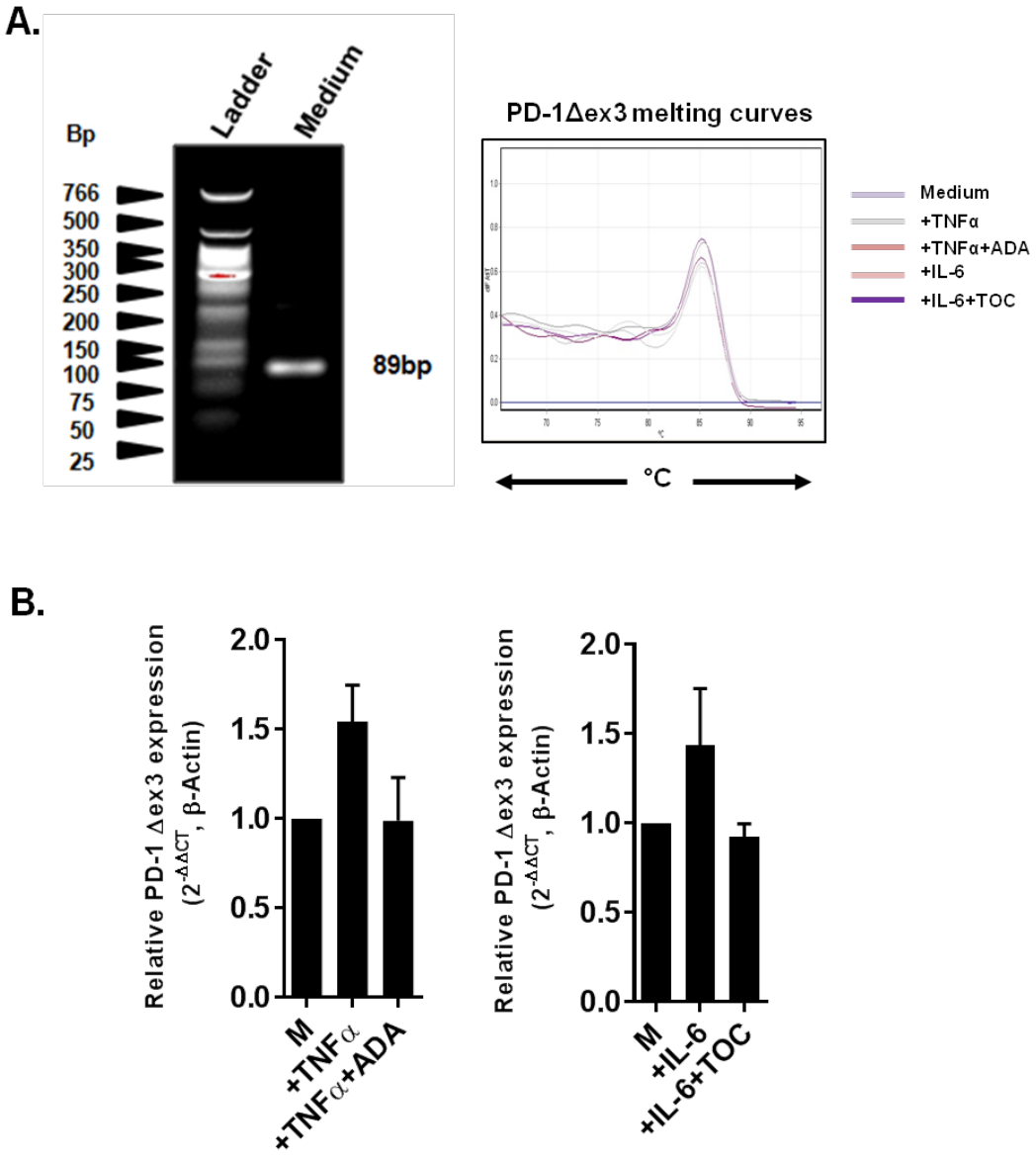

Figure 5.2 PD-1Δex3 is induced *in vitro* by TNFα and IL-6 in HC CD4+ T cells.
(A) Gel electrophoresis showing PD-1Δex3 primer specificity as demonstrated by a transcript of 89bp and by one-peak qPCR melting curves for medium only, +TNFα, +TNFα + ADA, +IL-6 and +IL-6 + TOC culture conditions. (B) Expression of PD-1Δex3 transcript in HC CD4+ T cells cultured for 5 days in the absence (medium, M) or presence of 10 ng/ml of TNFα (n=4) or IL-6 (n=3) ± anti-TNFα (adalimumab; ADA) or anti-IL-6R (tocilizumab; TOC) (all at 1 µg/ml). PD-1Δex3 expression was examined by qPCR and normalised to β-actin housekeeping gene (mean ± SEM).

Figure 5.3

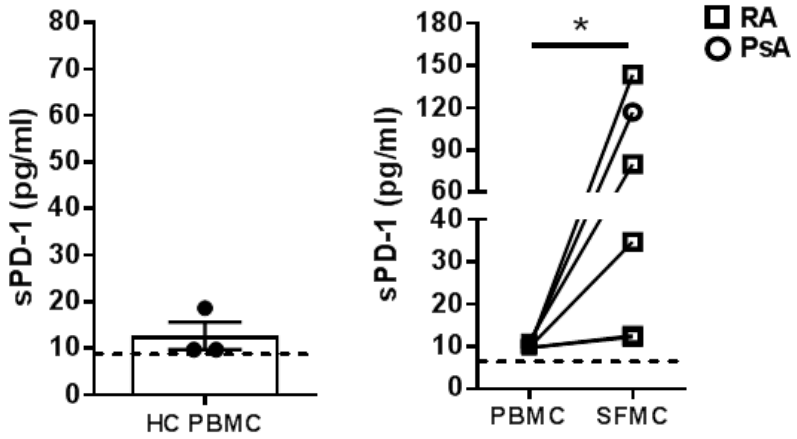

Figure 5.3 Soluble PD-1 (sPD-1) is higher in SFMC culture supernatants compared to PBMC culture supernatants.
Bulk PBMC from HC donors and bulk PBMC and SFMC from RA and PsA donors were cultured for 48 hrs in the presence of anti-CD3 mAb (OKT3; 100 ng/ml) (HC: n=3: RA: n=5, PsA: n=1). Day 2 supernatants were analysed by ELISA for sPD-1 levels. Data were analysed by Wilcoxon matched-pairs signed-rank test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5.2.2 Soluble PD-1 is detected in the serum and SF of both RA and PsA patients but not in HC serum and osteoarthritis (OA) serum and SF

The data so far demonstrated that both the PD-1Δex3 transcript and sPD-1 are induced in HC CD4+ T cells upon TNFα and IL-6 stimulation and that sPD-1 is detected in SFMC culture supernatants. Next, the presence of sPD-1 in the serum and SF of patients with inflammatory arthritis was investigated.

Two sets of cryopreserved RA and PsA serum and matched SF samples were analysed by ELISA for the presence of soluble PD-1. In the first sets of patient samples, sPD-1 was detected in 10/14 RA sera and in 11/14 RA SF. Similarly, sPD-1 was detectable in 8/10 PsA serum and SF samples (Figure 5.4). Soluble PD-1 levels for RA serum and SF were detected at ~26.8 and ~13.4 ng/ml respectively, while in PsA sPD-1 was detected at ~20.4 ng/ml for the serum, and ~11.9 ng/ml for the SF. Although sPD-1 was significantly lower in the PsA synovial fluid compared to the PsA serum, no significant differences were found when sPD-1 levels were compared between the two diseases. Serum levels of sPD-1 positively and significantly correlated with SF levels of sPD-1 in both RA and PsA (Figure 5.4C). These first sets (sets 1 & 2) were analysed with the main aim to detect sPD-1 and no investigation was performed on a control disease.

To address this and to further confirm the presence of sPD-1 in the serum and synovial fluid of inflammatory arthritis patients, two additional sets of RA and PsA patient samples (set 3 & 4) were selected. In the new experiments healthy serum and serum and SF from patients with osteoarthritis (OA) were selected and included in the analysis as controls. The two RA and PsA sets included patients not receiving TNF-inhibitor (TNFi) therapy, patients undergoing TNFi therapy and patients receiving methotrexate (MTX) therapy. In this new analysis, sPD-1 was significantly lower in

RA SF compared to RA PB and no significant difference was found between PsA serum and SF (Figure 5.5A). Soluble PD-1 was detectable in 0/2 HC serum, 0/3 OA serum and 0/4 OA SF samples, whilst it was detected at high levels in 13/17 RA and 13/18 PsA serum and SF samples. Soluble PD-1 levels for RA and PsA were detected as follows: ~15.3 ng/ml for RA Serum, ~10.5 ng/ml for RA SF, ~18.4 ng/ml for PsA serum and ~15.4 ng/ml for PsA SF (Figure 5.5B). As an extra internal control, the serum and paired SF from one representative OA sample were left untreated or spiked with 15 ng/ml of sPD-1fc. Results showed that in the spiked conditions sPD-1 was detected at 12.8 ng/ml and 20.8 ng/ml for the serum and SF, respectively (Figure 5.5C). In line with the analysis performed on the previous sets of patients, serum levels of sPD-1 positively and significantly correlated with SF levels of sPD-1 in both RA and PsA (Figure 5.5D).

Next, the levels of sPD-1 were investigated in the serum and SF of RA and PsA patients not treated with TNFi therapy versus patients treated with TNFi therapy. This was of interest because it was previously shown (see section 5.2.1) that the TNFi biologic adalimumab was able to abrogate the effect of TNFα on PD-1-mediated suppression of proliferation and to negatively modulate both PD-1Δex3 transcript and sPD-1 production in HC CD4+ T cell cultures. In this cross-sectional investigation, TNFi therapy-treated patients had lower levels of sPD-1 in both the serum and SF as compared to non-treated patients. Soluble PD-1 was detected at 15 ng/ml (serum) and at 11,2 ng/ml (SF) in patients not undergoing TNFi therapy. Conversely, sPD-1 levels in patients receiving TNFi therapy were detected at 2,1 ng/ml and 1,7 ng/ml for serum and SF, respectively (Figure 5.6A). Notably, the observed differences were more

pronounced and statistically significant in the RA group as compared to the PsA group (Figure 5.6B and 5.6C).

To further test whether TNFi therapy modulates sPD-1 levels, a preliminary longitudinal analysis was performed on 4 RA patients whose serum was collected before TNFi therapy (time 0) and at 6 and 12 months after initiation of TNFi therapy. Preliminary data showed that in 2/4 patients (patient #1 and patient #4), the serum levels of sPD-1 decreased over time upon treatment as compared to time 0. In patient #2, although time 0 was not available, sPD-1 was lower at 12 months as compared to 6 months while in patient #3 sPD-1 levels increased overtime following treatment (Figure 5.7). ELISA analysis was performed on the 4 RA serum samples to investigate whether the observed changes in sPD-1 levels were coupled with the modulation of proinflammatory cytokine TNFα and antiinflammatory cytokine IL-10, but neither TNFα nor IL-10 were detected in the tested sera (data not shown).

The levels of sPD-1 were also investigated in the serum and SF of RA and PsA patients not treated with MTX therapy versus patients treated with MTX therapy. Overall, patients undergoing MTX therapy had lower sPD-1 in the serum and SF compared to patients not treated with MTX therapy, although the observed differences were not significant (Figure 5.8A). Unexpectedly, data also revealed that in direct contrast with RA, the analysed PsA patients undergoing MTX therapy had higher sPD-1 levels in both the serum and SF as compared to patients not treated with MTX therapy (Figure 5.8B).

The presence of possible associations between cytokine levels, disease activity score and sPD-1 levels are of clinical interest. Although literature data is conflicting, it has been previously documented that sPD-1 levels correlate with certain cytokines in either the serum or SF of RA patients. The levels of the cytokines TNFα, IL-6 and IL-

1β in the serum and SF of RA and PsA patients (sets 3 and 4) as well as in OA and healthy controls were previously measured and data are shown in chapter 4.

Serum levels of sPD-1 positively correlated with serum levels of TNFα in the RA group (r^2 = 0.41; p=0.02) (Figure 5.9A) but not in the PsA group (Figure 5.9B) and no further correlations between IL-6 and IL-1β levels and sPD-1 levels were found. Soluble PD-1 levels were also evaluated against the disease activity score of 28 joints (DAS28), the tender joint count (TJC) or the swollen joint count (SJC) but no significant correlation was found (Figure 5.10 and Figure 5.11).

Figure 5.4

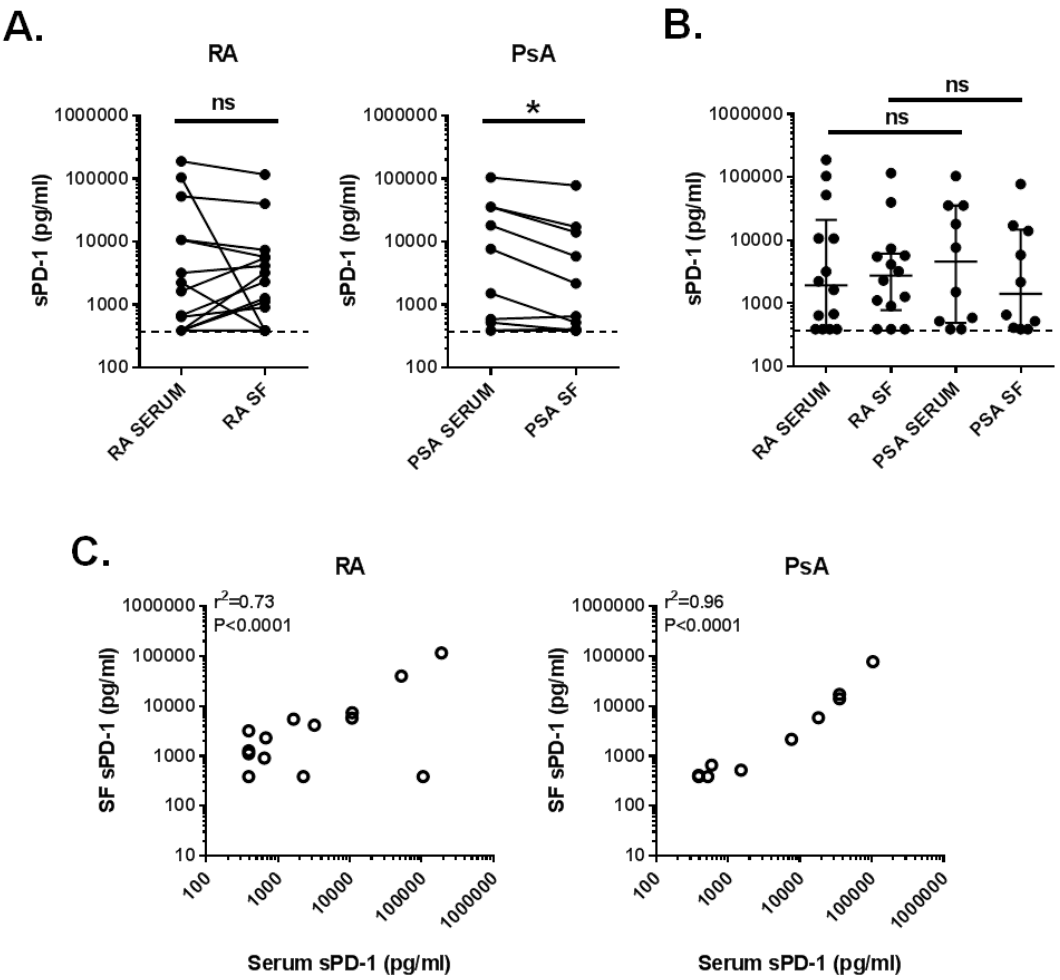

Figure 5.4 Soluble PD-1 (sPD-1) is detected in the serum and SF of RA and PsA patients.

(A-B) Soluble PD-1 levels in the serum and SF of RA and PsA samples (sets 1 and 2) were analysed by ELISA. The dashed line indicates the minimum detection limit and a $\log_{10}$ scale converted from a linear (pg/ml) scale is shown for each graph. (A) sPD-1 levels in paired serum and SF from RA (n=14) and PsA (n=10) patients. (B) sPD-1 levels comparison between serum and SF from RA and PsA patients (median ± IQR). (C) Correlation between serum and SF sPD-1 levels in RA (n=14) and PsA (n=10). Data in (A) were analysed by Wilcoxon matched-pairs signed-rank test. Data in (B) were analysed by Kruskal-Wallis test with Dunn's Multiple Comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data in (C) were analysed by Pearson test. ****$p < 0.0001$.

Figure 5.5

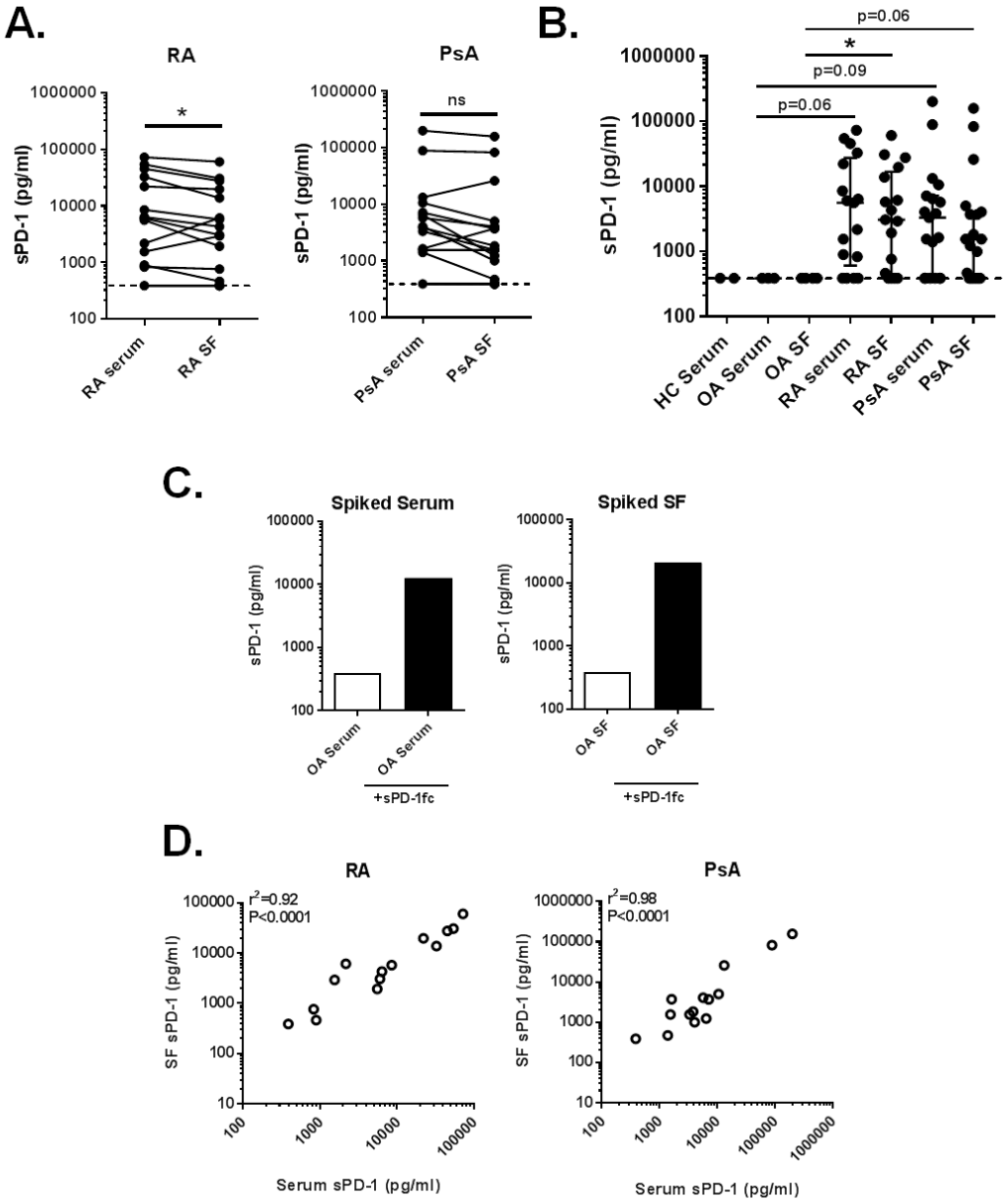

Figure 5.5 Soluble PD-1 (sPD-1) is detected at higher levels in the serum and SF of RA and PsA patients compared to healthy and OA controls.
(A-C) Soluble PD-1 levels in HC serum and in the serum and SF of OA, RA and PsA samples (sets 3 and 4) were tested by ELISA. The dashed line indicates the minimum detection limit and a $\log_{10}$ scale converted from a linear (pg/ml) scale is shown for each graph. (A) sPD-1 levels in paired serum and SF from RA (n=17) and PsA (n=18) patients. (B) sPD-1 comparison between HC, OA, RA and PsA serum and SF (HC, n=2; OA, n=3-4; RA, n=17; PsA, n=19) (median ± IQR). (C) sPD-1 levels in OA serum and SF without (white bars) and with (black bars) soluble PD-1fc (15 ng/ml) spike. (D) Correlation between serum and SF sPD-1 levels in RA (n=17) and PsA (n=19). Data in (A) were analysed by Wilcoxon matched-pairs signed-rank test while data in (B) were analysed by Kruskal-Wallis test with Dunn's Multiple Comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data in (D) were analysed by Pearson test. ****$p < 0.0001$.

Figure 5.6

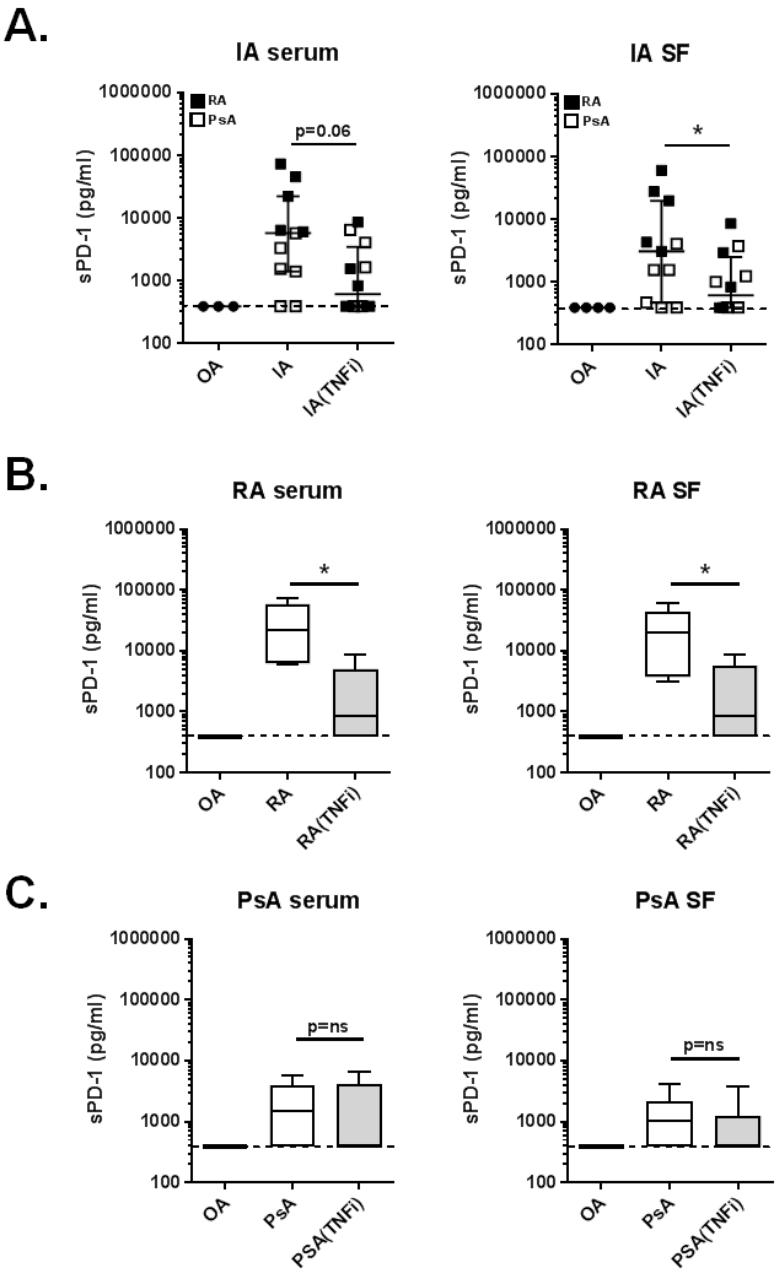

Figure 5.6 Soluble PD-1 (sPD-1) levels in the serum and SF of RA and PsA patients undergoing TNFi therapy are lower as compared to patients not receiving TNFi therapy. (A-C) Soluble PD-1 levels in the serum and SF of OA, RA and PsA patients (sets 3 and 4) were tested by ELISA. The dashed line indicates the minimum detection limit and a $\log_{10}$ scale converted from a linear (pg/ml) scale is shown for each graph. (A) Cross-sectional analysis of sPD-1 levels in RA and PsA paired serum/SF and OA serum/SF (OA, n=3-4; RA n=5, RA(TNFi) n=5, PsA n=6, PsA(TNFi) n=6) (median ± IQR). (B-C) Cross-sectional analysis of sPD-1 levels in RA (B) and PsA (C) serum and SF. Data (A-C) were analysed by Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 5.7

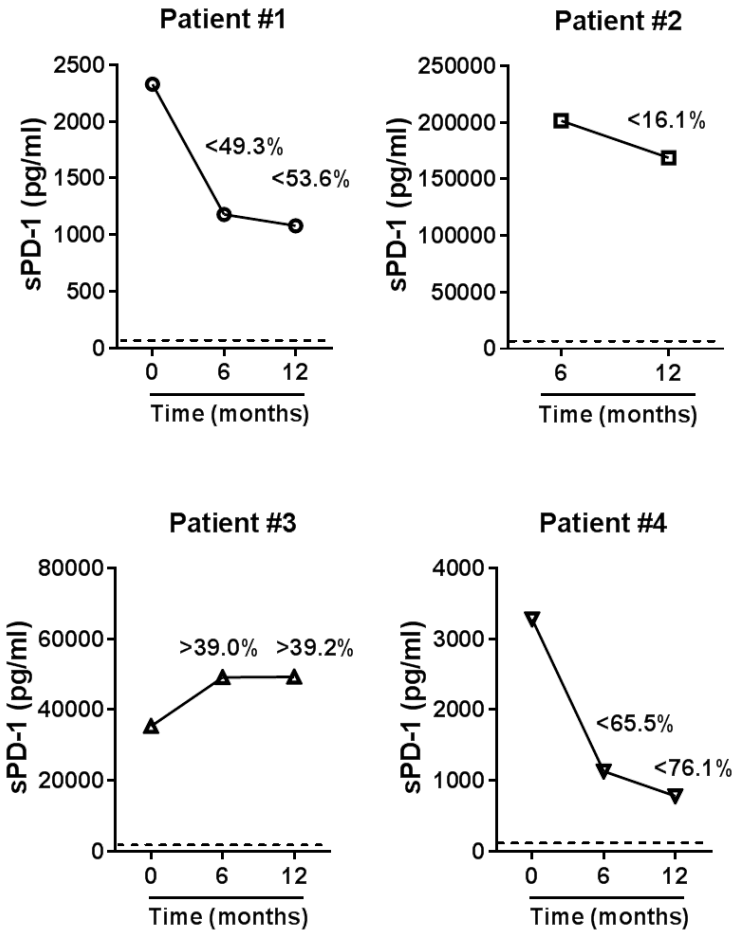

Figure 5.7 Soluble PD-1 (sPD-1) levels in the serum of RA patients undergoing TNFi therapy display variability overtime.
Longitudinal analysis of the levels of sPD-1 in the serum of RA patients undergoing TNFi therapy. sPD-1 was tested by ELISA at time 0 (no TNFi therapy; except for patient #2) and at 6 and 12 months after start of the therapy. The dashed line indicates the minimum detection limit and a linear scale is shown for each graph.

Figure 5.8

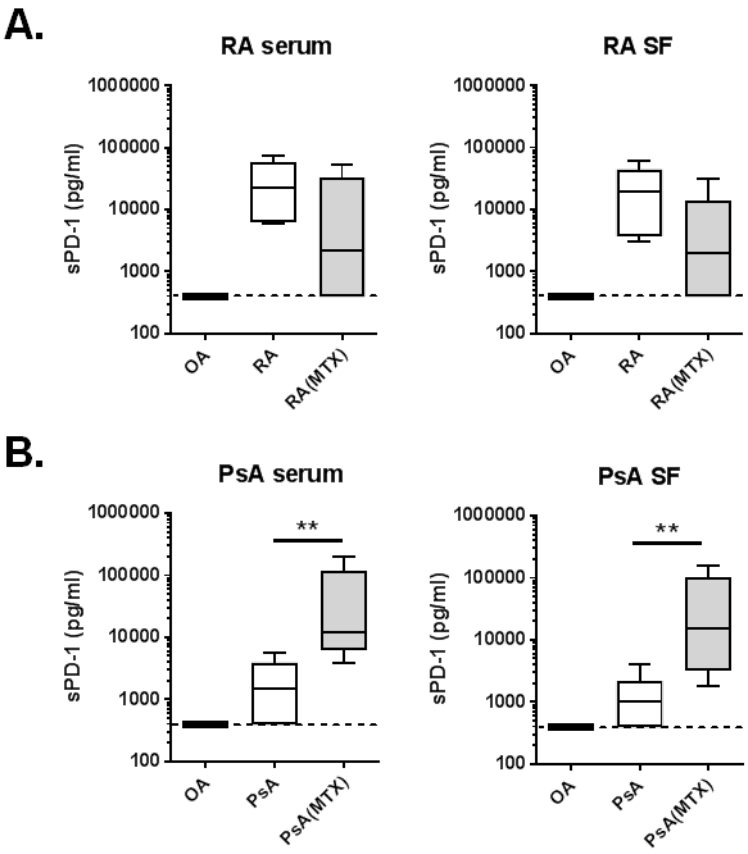

Figure 5.8 Soluble PD-1 (sPD-1) levels in the serum and SF of RA and PsA patients undergoing MTX therapy are lower and higher, respectively, as compared to patients not receiving MTX therapy.

(A-B) The levels of sPD-1 in the serum and SF of RA and PsA patients (sets 3 and 4) were analysed by ELISA. The dashed line indicates the minimum detection limit and a $\log_{10}$ scale converted from a linear (pg/ml) scale is shown for each graph. Cross-sectional analysis of sPD-1 levels in RA (A) and in PsA (B) paired serum and SF and OA serum and SF (OA n=3-4, RA n=5, RA(MTX) n=7; PsA n=6, PsA(MTX) n=6). Data (A-B) were analysed by Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 5.9

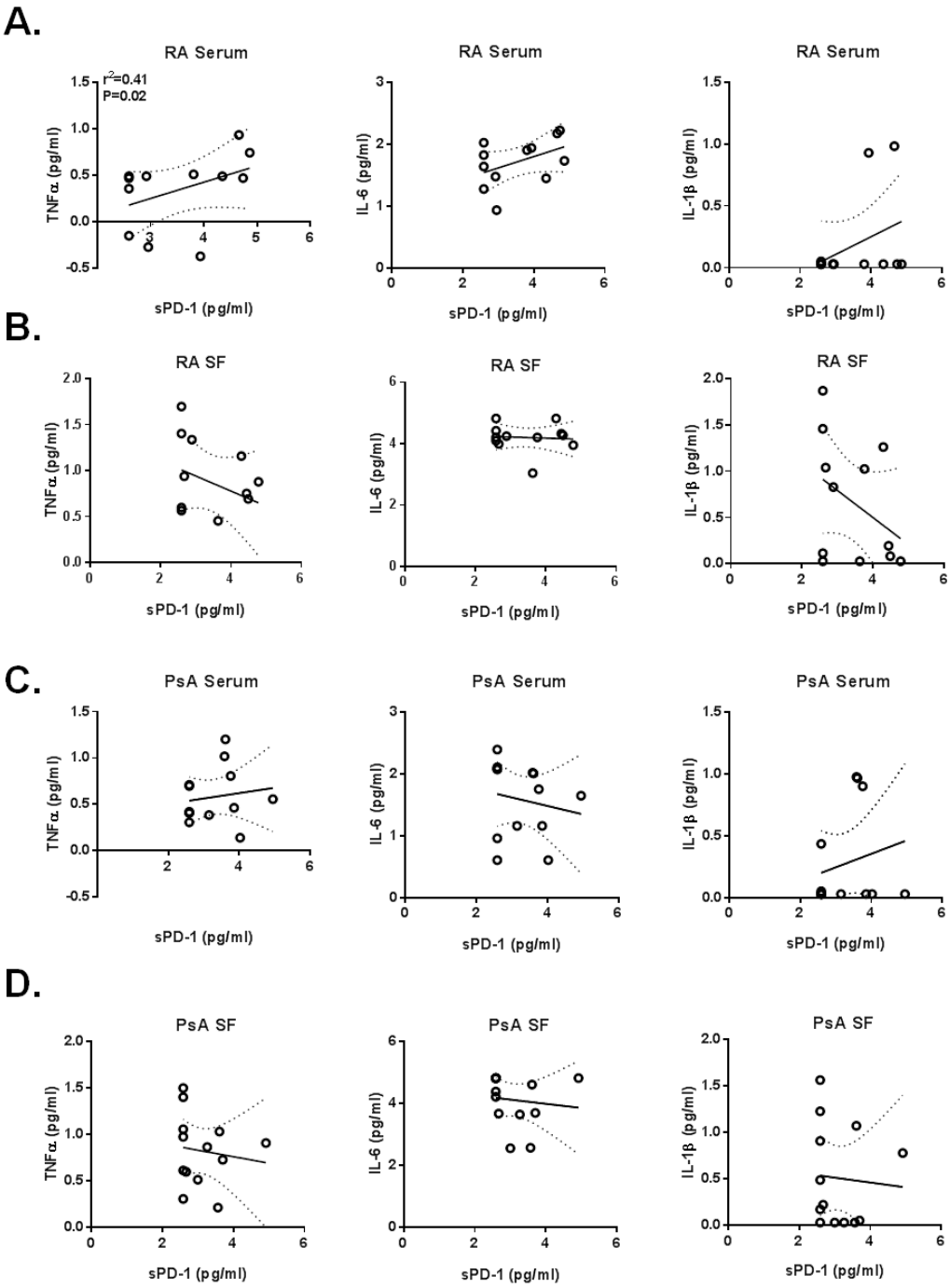

Figure 5.9 Correlations between soluble PD-1 (sPD-1) and proinflammatory cytokines levels in the serum and SF of RA and PsA patients.
(A-D) sPD-1 levels in RA serum n=12 (A), RA SF n=12 (B), PsA serum n=13 (C) and PsA SF n=13 (D) were assessed for correlation with the proinflammatory cytokines TNFα, IL-6 and IL-1β by Spearman's test. Symbols represent individual patients. Individual data were transformed in Log_{10} for each group. Linear scales are shown.

Figure 5.10

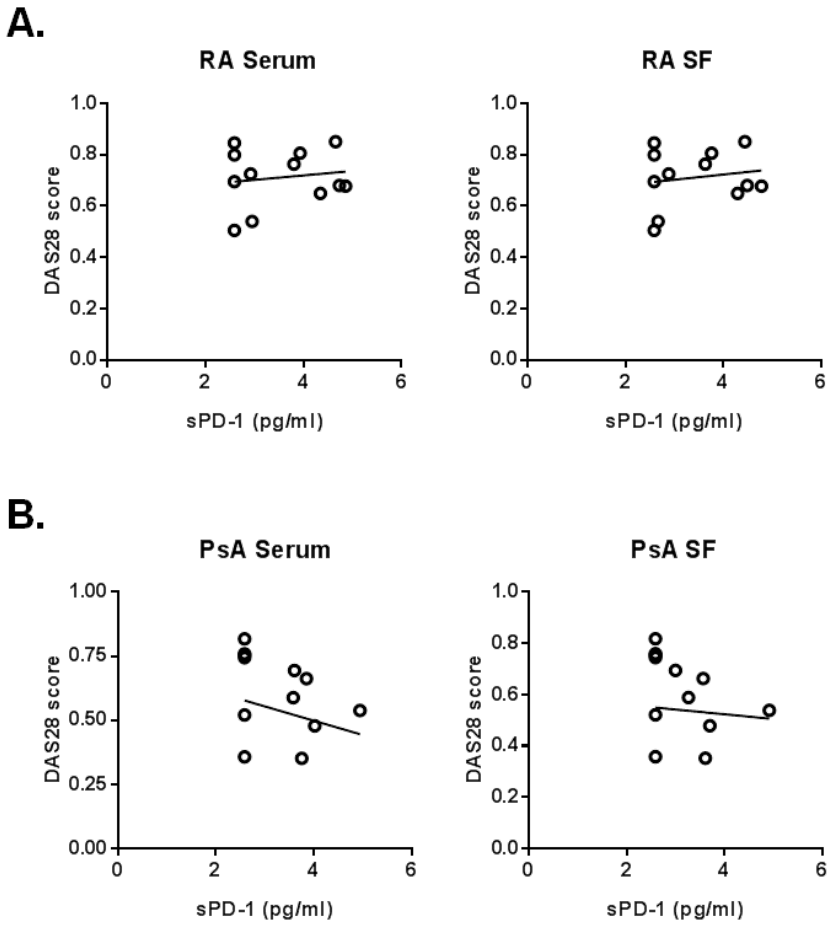

Figure 5.10 Correlations between soluble PD-1 (sPD-1) levels in the serum and SF of RA and PsA patients and the disease activity score in 28 joints (DAS28).
(A-B) sPD-1 levels in the RA serum n=12 and RA SF n=12 (A) and in PsA serum n=13 and PsA SF n=13 (B) were assessed for correlation with the disease activity score in 28 joints (DAS28) by Spearman's test. Symbols represent individual patients. Individual data were transformed in Log_{10} for each group. Linear scales are shown.

Figure 5.11

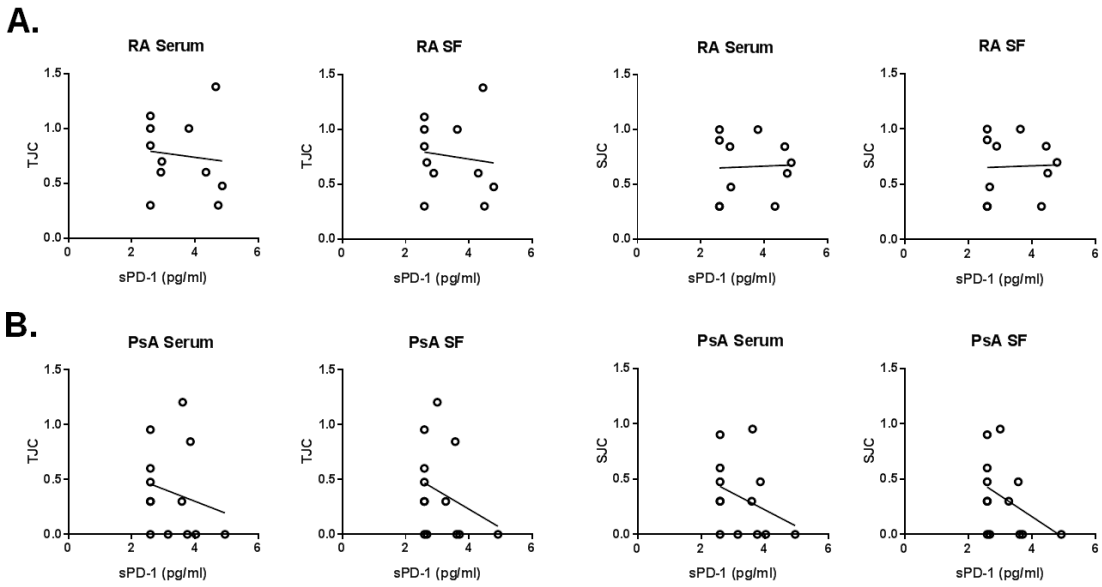

Figure 5.11 Correlations between soluble PD-1 (sPD-1) levels in the serum and SF of RA and PsA patients and the tender join count (TJC) or swollen joint count (SFJ).
(A-C) sPD-1 levels in the RA serum n=11 and SF n=11 (A) and in the PsA serum n=12 and SF n=12 (B) were assessed for correlation with the tender joint count (TJC) or the swollen joint count (SJC) parameters by Spearman's test. Symbols represent individual patients. Individual data were transformed in Log_{10} for each group. Linear scales are shown.

5.2.3 Soluble PD-1 modulates PD-1-mediated suppression of HC CD4+ T cells and induces proliferation in CD4+ T cell:CD14+ monocyte co-cultures

Data so far have shown that sPD-1 is induced by proinflammatory cytokines typically detected in RA and PsA, it is found mainly in the supernatants of RA and PsA SFMC cultures and it can be detected in the serum and SF of patients with RA or PsA. The next experiments were performed to investigate whether sPD-1 was able to negatively modulate PD-1-mediated suppression of proliferation *in vitro*.

To investigate this, a human PD-1fc chimera was used to mimic the effect of naturally occurring sPD-1. First, sPD-1fc was tested for its effect on T cell proliferation of anti-CD3 stimulated HC CD4+ T cells cultured in absence of PD-L1fc. Soluble PD-1fc was added at the beginning of the culture at two increasing concentrations (0.5 and 1 μg/ml) and cells were cultured for 5 days. In these experiments, sPD-1fc did not significantly increase nor decrease T cell proliferation as compared to medium only condition suggesting no direct effect on HC CD4+ T cells cultured without the presence of accessory cells (Figure 5.12A).

Next, HC CD4+ T cells were cultured in plates pre-coated with increasing amounts of PD-L1fc ligand and in the absence or presence of sPD-1fc chimera (0.5 or 1 μg/ml). In PD-L1fc pre-coated plates, addition of sPD-1fc was able to abrogate the effect of the ligand in a dose-dependent fashion resulting in a less efficient suppression of T cell proliferation compared to medium only (Figure 5.12B, 5.12C).

In absence of sPD-1fc, the average level of suppression of proliferation in the 0.1 and 1 μg/ml PD-L1fc conditions was ~20% and ~32%, respectively, as compared to medium only (0 μg/ml). In presence of 0.5 μg/ml of sPD-1fc, suppression of proliferation decreased to an average of ~6% and ~18%, respectively. No suppression of proliferation (~0%) was found when 1 μg/ml of sPD-1fc was added to the cell

cultures (Figure 5.12B, 5.12C). These data indicate that in a HC CD4+ T cell-only culture system, sPD-1 is able to negatively modulate an otherwise functional PD-1/PD-L1 interaction.

To further investigate the role of sPD-1 during PD-1:PD-L1 interaction, a co-culture system using HC CD4+ T cells and autologous CD14+ cells as a source of natural PD-L1 was set up. Firstly, the ability of HC CD14+ cells to express PD-L1 was analysed by flow cytometry. HC CD14+ cells were isolated from HC PBMC and cultured overnight with either IFN-γ, a known inducer of PD-L1 (59, 324), or proinflammatory cytokines IL-6, TNFα and a combination of IL-6 + TNFα. These proinflammatory cytokines have also been described as inducers of PD-L1 expression (394-396). After 12 hours in culture, the majority of IFN-γ-treated cells were PD-L1+ as compared to cells left unstimulated (Medium: ~18% of CD14+PD-L1+ cells; IFN-γ: ~98% of CD14+PD-L1+ cells). This was also the case when cells were stimulated with IL-6, TNFα or with a combination of IL-6 + TNFα (Figure 5.13A, 5.13B). TNFα was a stronger inducer of PD-L1 as compared to IL-6 (Medium: ~5%; IL-6: ~30%; TNFα: ~70% of CD14+PD-L1+ cells) and the two cytokines showed a synergistic effect when used together (IL-6 + TNFα: ~90% of CD14+PD-L1+ cells). These data indicate that, *in vitro,* HC CD14+ cells are able to upregulate PD-L1 following cytokine stimulation.

Next, freshly isolated HC CD4+ T cells and autologous monocytes were cultured at a 1:1 ratio, with soluble anti-CD3 mAb (100 ng/ml) in the absence or presence of increasing doses of sPD-1fc or IgG1fc control (0.25, 0.5 and 1 μg/ml).

Addition of sPD-1fc led to a dose-dependent increase in T cell proliferation compared to control-treated cells. The observed increase in proliferation was statistically significant in presence of 1 μg/ml of sPD-1fc (Figure 5.14A, 5.14B).

Importantly, no variation in proliferation was observed in presence of the specific IgG1fc control. These results were confirmed in additional experiments in which HC CD4+ T cells were cultured with autologous CD14+ monocytes at a 1:0.5 ratio (Figure 5.14C) and in preliminary experiments with HC CD8+ T cells cultured with autologous CD14+ monocytes at 1:1 ratio (Figure 5.14D). In these experiments HC CD8+ T cell proliferation increased to an average of ~37% in presence of 1 μg/ml sPD-1fc as compared to medium only (Figure 5.14D).

These data indicate that soluble PD-1fc used to mimic natural soluble PD-1 modulates PD-1 ligation in both an artificial system (PD-L1fc pre-coated plates) as well as in a more physiological context (in presence of APCs capable of upregulating PD-L1).

Figure 5.12

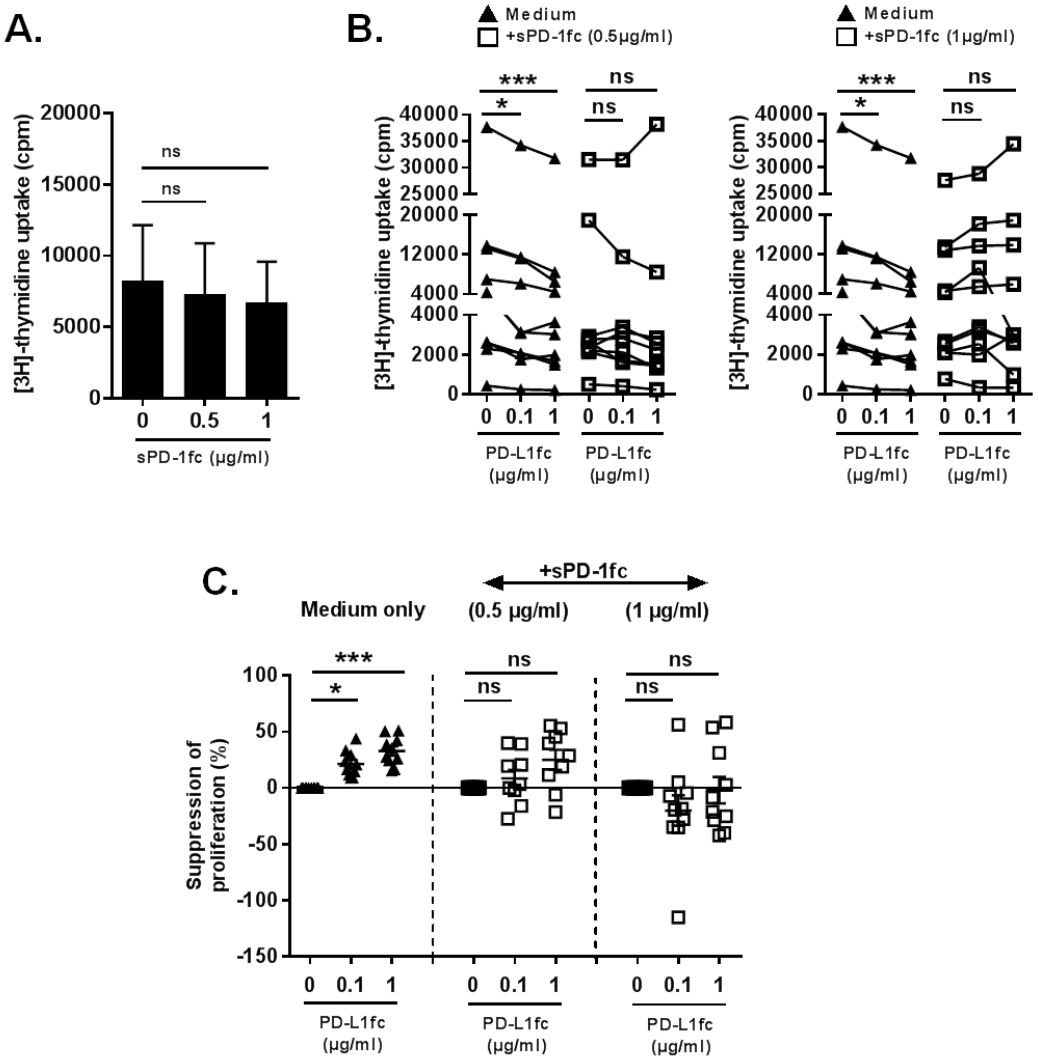

Figure 5.12 Soluble PD-1 (sPD-1) modulates PD-1-mediated suppression of HC CD4+ T cells.

(A) HC CD4+ T cells (n=9) were cultured with immobilised anti-CD3 mAb (OKT3) in the presence of increasing concentrations of sPD-1fc (0, 0.5 and 1 µg/ml). Proliferation was assessed at day 5 by [^{3}H]-thymidine incorporation and displayed as count per minute (cpm). (B) Proliferation and (C) suppression of proliferation of HC CD4+ T cells cultured in anti-CD3 mAb (OKT3) and PD-L1fc pre-coated plates with or without sPD-1fc (0.5 and 1µg/ml) (n=9-10). Data were analysed by Friedman Test with Dunn's Multiple Comparison test. *$p < 0.05$ and ***$p < 0.001$.

Figure 5.13

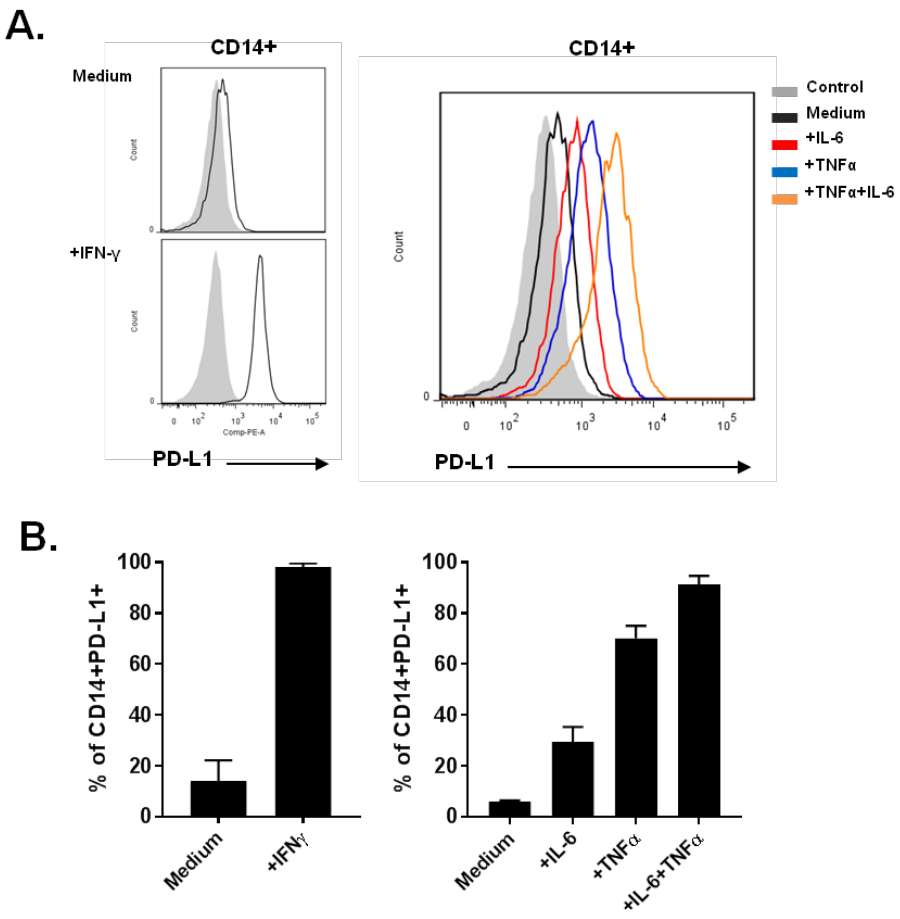

Figure 5.13 PD-L1 expression in HC CD14+ monocytes increases with IFN-γ, IL-6 and TNFα cytokine stimulation.
CD14+ monocytes where positively isolated from HC PBMC and cultured overnight at 37 ºC in medium only or in medium supplemented with IFN-γ, IL-6, TNFα or a mix of IL-6 and TNFα (All cytokines used at 10 ng/ml). PD-L1 expression was assessed after 12 hrs by flow cytometry. (A) Representative experiments. Shaded histograms represent the isotype control, open histograms indicate the expression profile for PD-L1 with/without the specific cytokine stimulation. (B) Cumulative data showing the percentage of monocytes expressing PD-L1 (n=3 for IFN-γ; n=2 for IL-6, TNFα and IL-6+TNFα conditions).

Figure 5.14

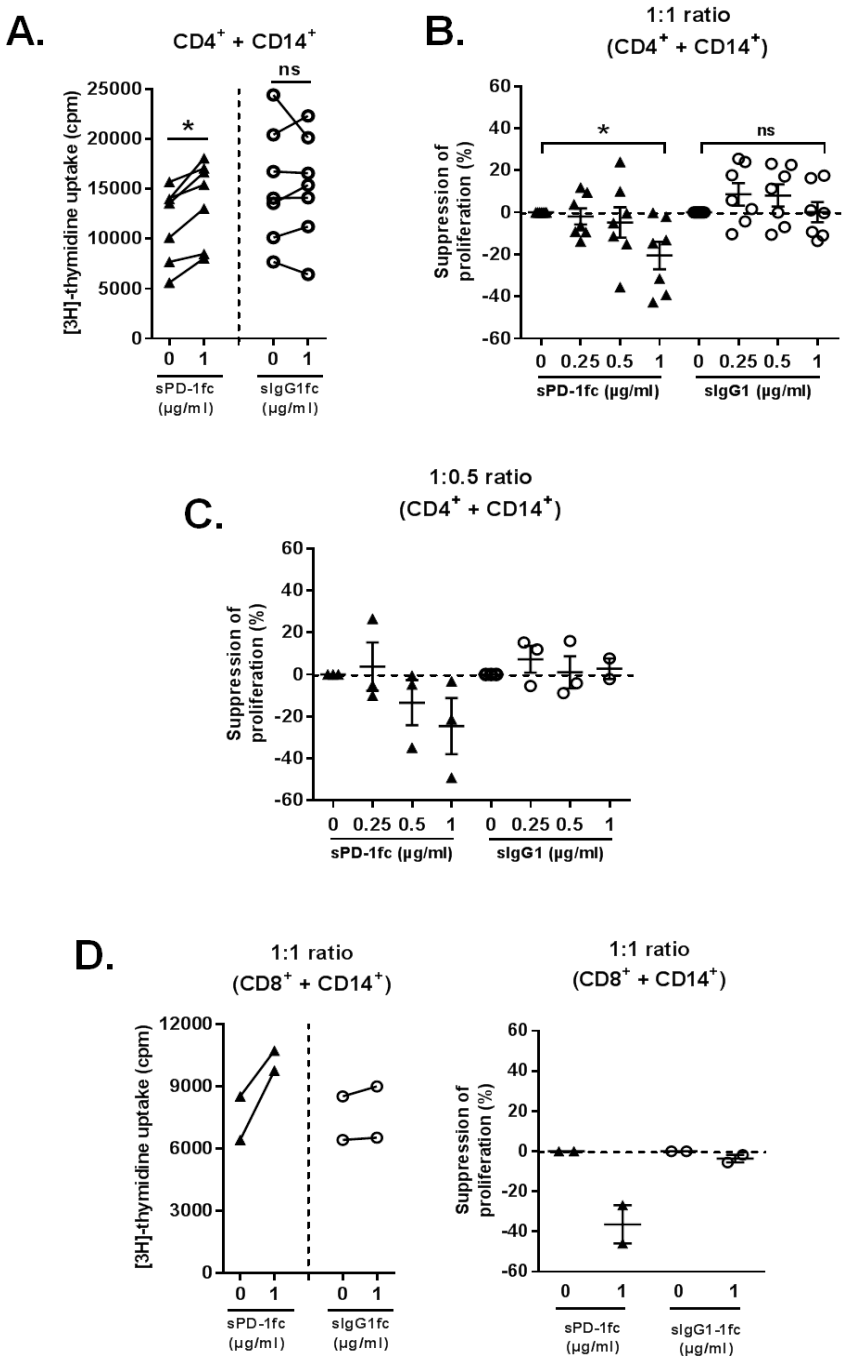

Figure 5.14 sPD-1 induces proliferation in HC CD4+ and CD8+ T cell/CD14+ monocyte co-cultures.

Proliferation (A) and suppression of proliferation (B-C) of HC CD4+ T cells cultured for 5 days with autologous CD14+ monocytes at a 1:1 ratio (n=7) and 1:0.5 ratio (n=3, except sIgG1; 1 µg/ml, n=2) in the presence of soluble anti-CD3 mAb (100 ng/ml) and soluble PD-1fc/IgG1fc control (0, 0.25, 0.5 and 1 µg/ml). (D) Proliferation and suppression of proliferation of HC CD8+ T cells cultured with autologous CD14+ monocytes at 1:1 ratio (n=2) in the presence of soluble anti-CD3 mAb (100 ng/ml) and soluble PD-1fc/IgG1fc control (1 µg/ml). Data were analysed by Wilcoxon signed rank test (A-B). *$p < 0.05$ and ***$p < 0.001$.

5.2.4 Soluble PD-1 does not modulate the regulatory function of HC CD4+CD25+ Treg cells *in vitro*

Data so far have shown that a sPD-1fc chimera, used to mimic sPD-1, can negatively modulate PD-1 ligation in CD4+ T cell-only cultures and in CD4+:CD14+ co-cultures but it is unclear whether or not sPD-1 has also a role in modulating Treg-mediated regulation. This is of interest because PD-L1-mediated signalling in PD-1+ induced regulatory T cells (iTregs) has been shown to increase the protein expression of forkhead box 3 (FoxP3) leading to enhanced suppressive functions (98, 394). Hence, sPD-1 could potentially act by disrupting PD-1/PD-L1 interaction influencing Treg-mediated suppression.

To investigate this further, an *in vitro* Treg cell suppression assay was set up. An initial optimization of the assay was performed to evaluate the ability of HC Treg cells to regulate T effector (Teff) cells proliferation. HC PB-derived Treg ($CD4+CD25+CD127^{low}$) cells were positively isolated and cultured with autologous cell trace violet (CTV)-stained PBMC (Teff cells) in the presence of anti-CD3 mAb (OKT3; 100 ng/ml) or with anti-CD3/CD28 activation beads (1 bead/5 cells). Teff cells were cultured with Treg cells at 3 different Teff:Treg cell ratios (1:0, 1:0.25 and 1:0.5) and proliferation of CD4+ and CD8+ cells within the Teff population was assessed in parallel experiments on day 3 by gating on CTV+ populations. A representative plot showing Treg cells purity in two representative HC donors and the gating strategy of the Treg suppression assay is shown in Figure 5.15A and Figure 5.15B.

In these initial experiments, the presence of Treg cells led to a cell ratio–dependent reduction in the proliferation of autologous CD4+ and CD8+ cells (Figure 5.16A and 5.16B). Cell proliferation in absence of Treg cells was higher in beads-

stimulated cultures (Figure 5.16B) compared to anti-CD3-stimulated cultures (Figure 5.16A). Similarly, the suppressive capacity of Treg cells was higher in beads-stimulated cultures compared to anti-CD3-stimulated cultures showing percentages of suppression of ~70% and ~80% compared to ~40% and ~60% (ratios 1:0.25 and 1:0.5, respectively). As readout for Treg cells ability to control cell activation, PD-1+ cell percentages and PD-1 medium intensity fluorescence (MFI) were also evaluated in a group of Teff cells cultured with or without Treg cells in presence of anti-CD3 (OKT3) stimulation or anti-CD3/CD28 beads.

PD-1 expression was consistently increased in both CD4+ and CD8+ T cells upon cell activation and in absence of Treg cells (Figure 5.17A and Figure 5.18A). On the contrary, addition of Treg cells to the cultures consistently reduced the percentage of PD-1+CD4+ and PD-1+CD8+ cells as well as PD-1 MFI in a dose-dependent fashion (Figure 5.17B and 5.17C and Figure 5.18B and 5.18C). Teff cell, instead of Treg cells, were then added to the cultures (1.5:0 ratio) to control for cell density, and no difference in PD-1+ percentages or PD-1 MFI were found as compared to 1:0 condition (Figure 5.17 and 5.18).

These results demonstrated that the suppression assay was functional and that HC PB-derived Treg cells suppressed proliferation of autologous Teff cells despite the different methods used for activation. The data further demonstrated that Treg cells can modulate Teff cell activation as shown by decreased percentages of PD-1+ Teff cells and decreased PD-1 MFI.

Next, further experiments were set up to investigate whether the presence of sPD-1fc added from the start of the culture could modulate Treg-mediated regulation

of proliferation and cytokine production. Firstly, the proliferation of Teff cells cultured in absence of Treg cells and with sPD-1fc at 1 and 2 μg/ml was evaluated.

Unexpectedly, in these experimental conditions, sPD-1fc had no effect on proliferation as shown by similar percentages detected across all conditions for both CD4+ and CD8+ cells (Figure 5.19A, 5.19B). Teff cells were then cultured in presence of Treg cells at 3 different cell ratios in absence or presence of sPD-1fc or sIgG1 control. Data show that in all conditions Treg cells led to a ratio-dependent reduction in the proliferation of CD4+ and CD8+ cells and that addition of sPD-1fc during the co-culture, had no positive or negative effect on their ability to suppress Teff cells proliferation (Figure 5.19C, 5.19D).

Next, co-culture supernatants were tested for the presence of cytokines IFN-γ and TNFα. In line with the proliferation data, activation with anti-CD3/CD28 beads was able to induce higher production of both cytokines as compared to activation with anti-CD3 only. Furthermore, Treg cells strongly suppressed the secretion of IFN-γ and TNFα in a cell ratio-dependent fashion but the levels of suppression observed were not significantly different between medium only and +sPD-1fc or +IgG1 control conditions (Figure 5.20A, 5.20B).

These data indicate that in the selected experimental conditions, soluble PD-1 is unable to negatively or positively modulate Treg-mediated suppression of proliferation and cytokine production.

Figure 5.15

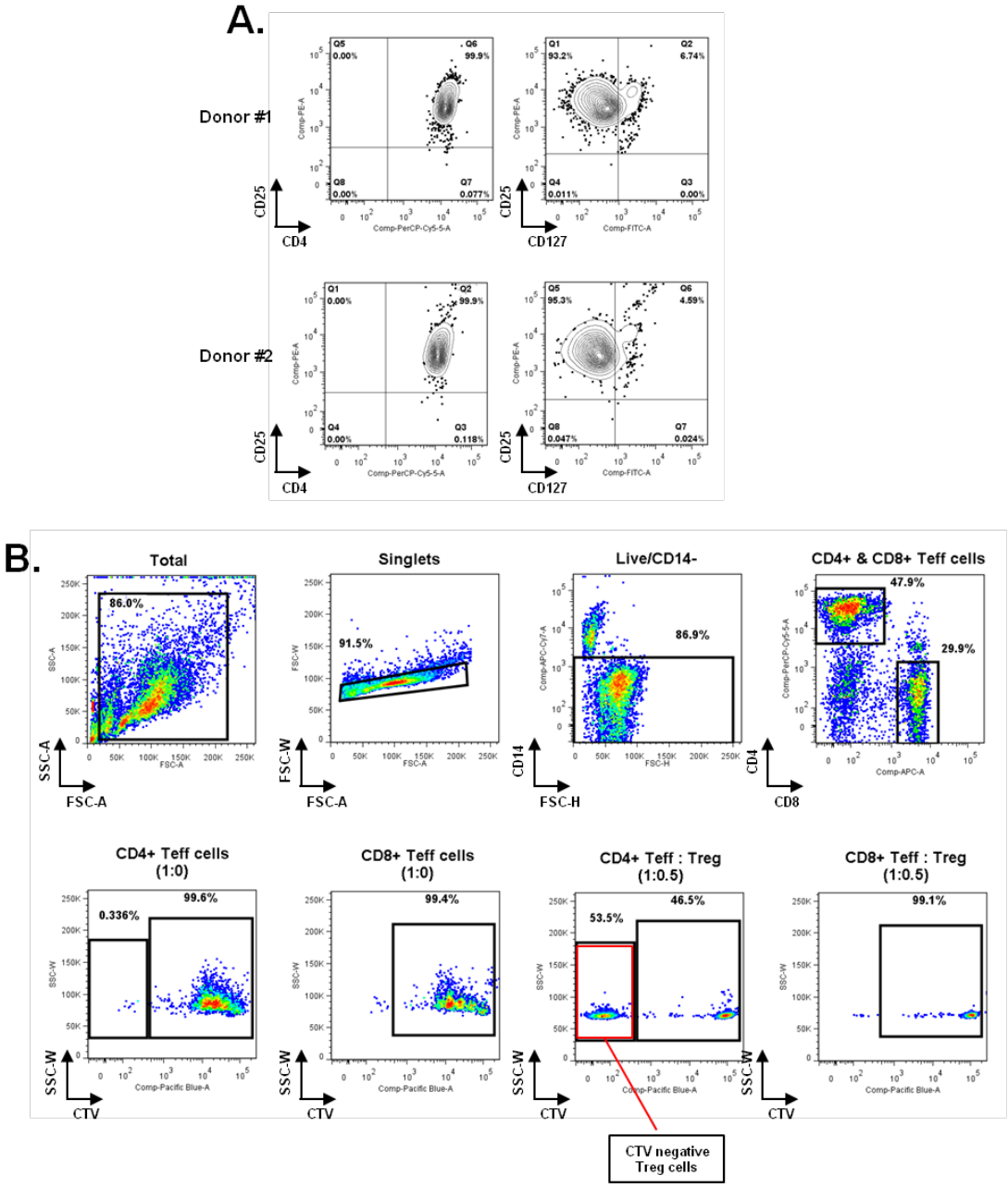

Figure 5.15 Gating strategy for the analysis of the Treg cell suppression assay.
(A) Purity staining of PB-derived Treg cells from two representative HC donors showing that ,phenotypically, isolated cells are CD4+CD25+ and CD4+CD127low. (B) Gating strategy for the Treg suppression assay. (B) FACS plots from a representative donor stimulated with anti-CD3/CD28 beads and analysed at day 3. Cells from Treg:Teff co-cultures were gated and analysed as follows: First total cells, followed by singlets, live/CD14- cells and finally CD4+ versus CD8+ cells. The dilution of the CTV dye was assessed for CD4+ and CD8+ cells in Teff alone (1:0) and Teff + Treg (1:0.5) co-cultures.

Figure 5.16

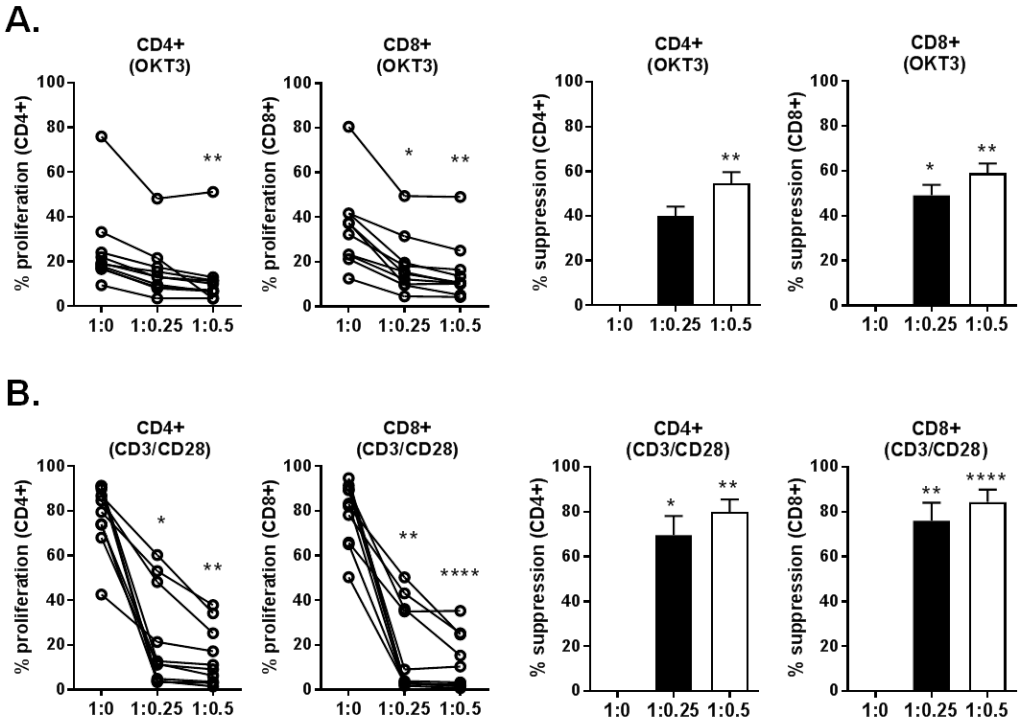

Figure 5.16 HC CD4+CD25+ Treg cells are able to suppress autologous CD4+ and CD8+ cell proliferation.

HC PBMC (Teff cells) were labelled with cell trace violet (CTV) and cultured in the presence of anti-CD3 monoclonal antibody (100 ng/ml) (A) or anti-CD3/CD28 beads (1 bead every 5 cells) (B) ± autologous PB CD4+CD25+ Treg cells (added at the indicated Teff:Treg cell ratios). On day 3, cell proliferation was assessed by gating on CD4+ and CD8+ cells within PBMC Teff cells. (A-B) Cumulative data showing the percentage of proliferating cells and the percentage of suppression in the absence or presence of Treg cells (n=10). The percentage of Teff proliferation is shown for each Teff:Treg cell ratio. Bars show the mean ± SEM. Data were analysed by Kruskal-Wallis test with Dunn's multiple comparison test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Figure 5.17

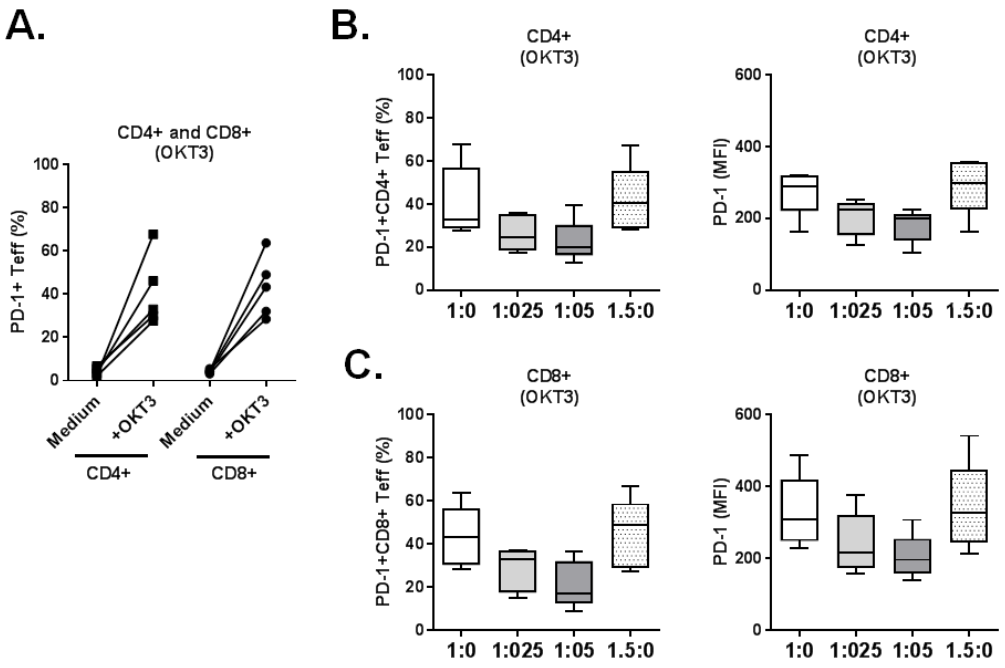

Figure 5.17 HC CD4+CD25+ Treg cells suppress PD-1 expression in OKT3-stimulated autologous Teff cells.

HC PBMC (Teff cells) were labelled with cell trace violet (CTV) and cultured in the presence of anti-CD3 monoclonal antibody (100 ng/ml) ± autologous PB CD4+CD25+ Treg cells (added at the indicated Teff:Treg cell ratios). On day 3, PD-1 expression was assessed by gating on CD4+ and CD8+ cells within PBMC Teff cells. (A) PD-1+ cells within CD4+ and CD8+ cells cultured in absence (medium) or presence (+OKT3) of TCR stimulation and in absence of Treg cells (n=5). (B-C) PD-1+ cell percentage (%) and PD-1 medium intensity fluorescence (MFI) of CD4+ and CD8+ cells cultured without/with autologous CD4+CD25+ Treg cells (n=5).

Figure 5.18

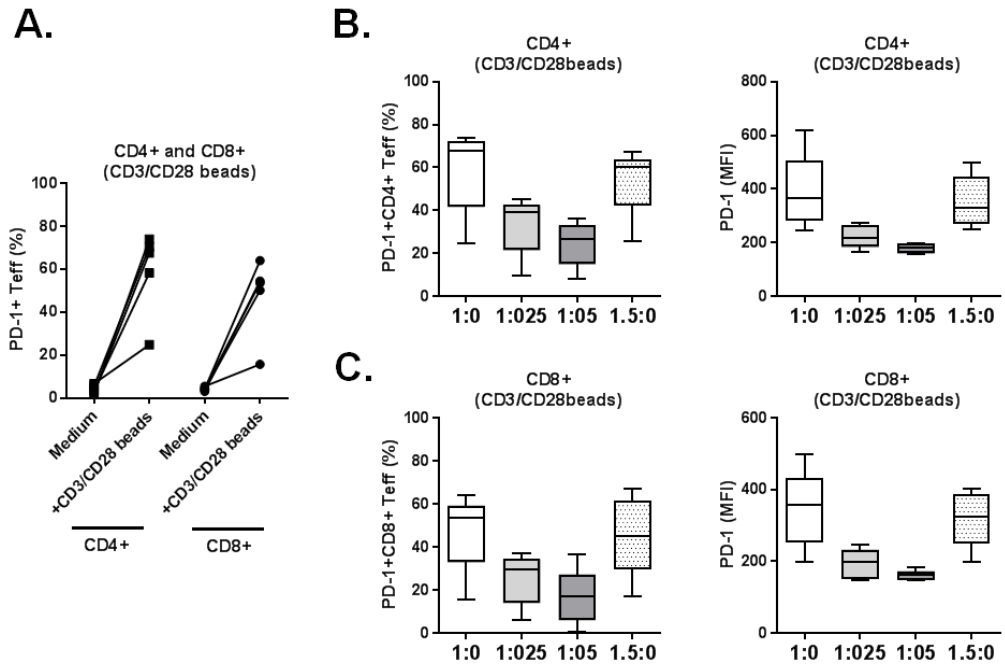

Figure 5.18 HC CD4+CD25+ Treg cells suppress PD-1 expression in anti-CD3/CD28 bead-stimulated autologous Teff cells.
HC PBMC (Teff cells) were labelled with cell trace violet (CTV) and cultured in the presence of anti-CD3/CD28 activation beads (1 bead every 5 cells) ± autologous PB CD4+CD25+ Treg cells (added at the indicated Teff:Treg cell ratios). On day 3, PD-1 expression was assessed by gating on CD4+ and CD8+ cells within PBMC Teff cells. (A) PD-1+ cells within CD4+ and CD8+ cells cultured in absence (medium) or presence (+OKT3) of TCR stimulation and in absence of Treg cells (n=5). (B-C) PD-1+ cell percentage (%) and PD-1 medium intensity fluorescence (MFI) of CD4+ and CD8+ cells cultured with/without autologous CD4+CD25+ Treg cells (n=5).

Figure 5.19

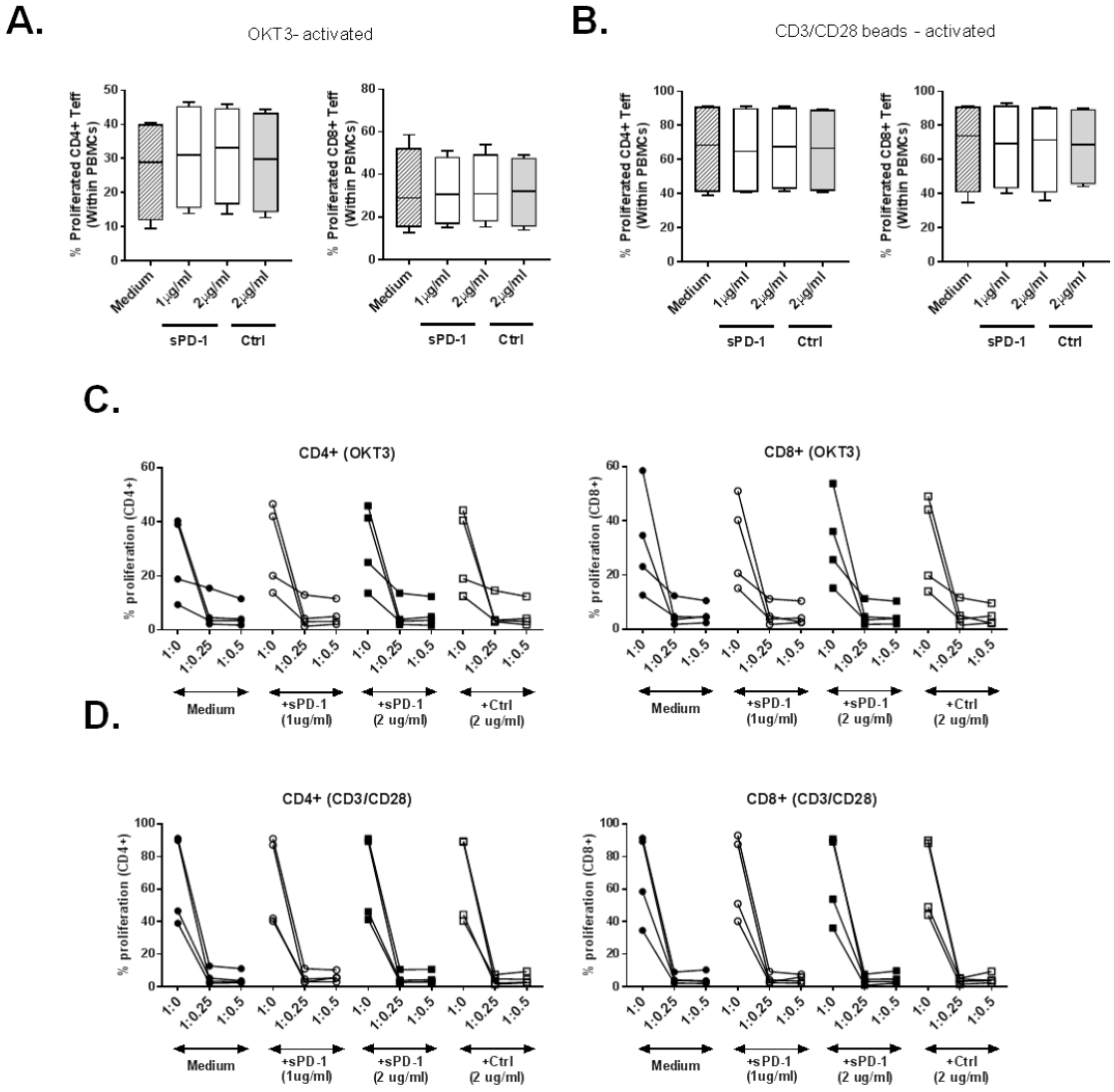

Figure 5.19 HC CD4+CD25+ Treg cell-mediated suppression of proliferation is not modulated by sPD-1.

HC PBMC (Teff cells) were labelled with cell trace violet (CTV) and cultured in the presence of anti-CD3 monoclonal antibody (100 ng/ml) or anti-CD3/CD28 beads (1 bead every 5 cells), ± autologous CD4+CD25+ Treg cells (added at the indicated Teff:Treg cell ratios) and ± sPD-1fc (1 and 2 µg/ml) or sIgG1fc (Ctrl: 2 µg/ml). On day 3, cell proliferation was assessed by gating on CD4+ and CD8+ cells within PBMC Teff cells. (A-B) Effect of sPD-1 or sIgG1 control on Teff proliferating cells stimulated with (A) anti-CD3 monoclonal antibody or (B) anti-CD3/CD28 beads in absence of Treg cells. (C-D) Cumulative data showing the percentage of proliferating CD4+ and CD8+ cells (within PBMC) in presence of Treg cells (n=4). Data were analysed by Kruskal-Wallis test with Dunn's multiple comparison test.

Figure 5.20

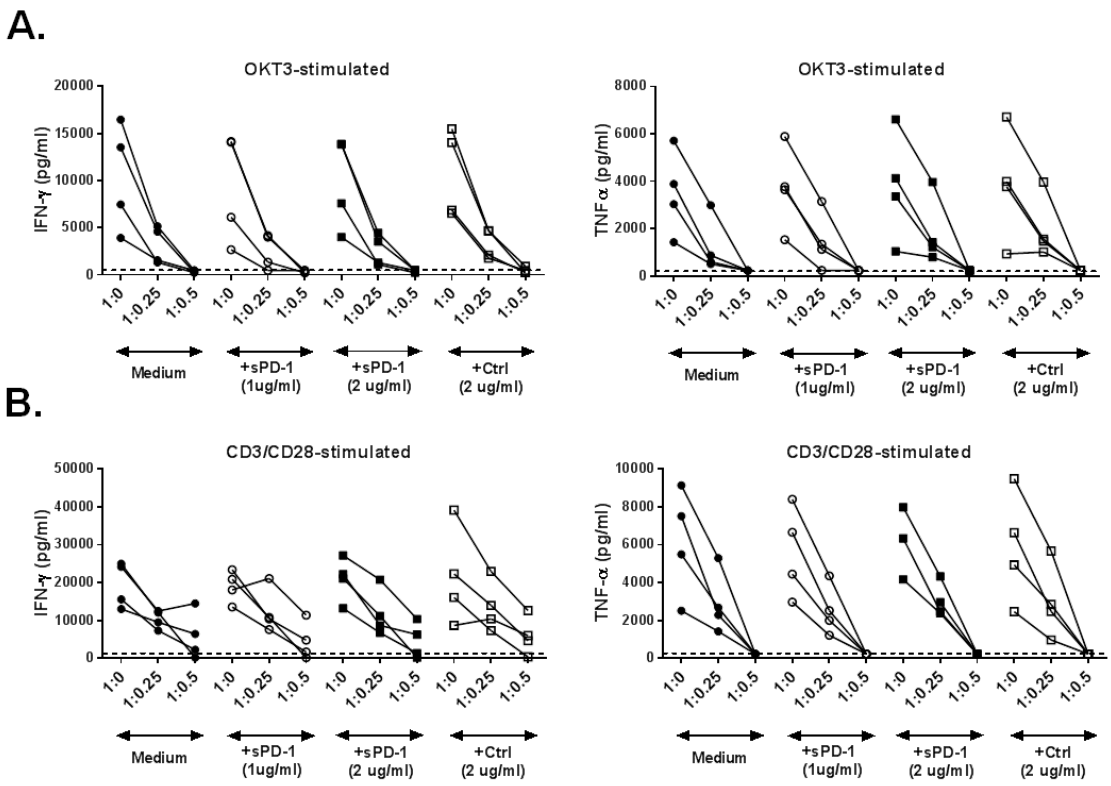

Figure 5.20 HC CD4+CD25+ Treg cell-mediated suppression of IFN-γ and TNFα production is not modulated by sPD-1.
HC PBMC (Teff cells) were labelled with cell trace violet (CTV) and cultured in the presence of (A) anti-CD3 monoclonal antibody (100 ng/ml) or (B) anti-CD3/CD28 beads (1 bead every 5 cells), ± autologous PB CD4+CD25+ Treg cells (added at the indicated Teff:Treg cell ratios) and ± PD-1fc (1 and 2 µg/ml) or IgG1fc (2 µg/ml). On day 3, cell culture supernatants were collected and analysed by ELISA for the presence of IFN-γ and TNFα. The broken horizontal line in A and B indicates the lower limit of detection.

5.3 Discussion

The data presented in this chapter provide new findings regarding the production of sPD-1 and its biological activity. Firstly, it was demonstrated that TNFα or IL-6 added to HC CD4+ T cells induce soluble PD-1 production and that the biologics adalimumab (anti-TNFα) and tocilizumab (anti-IL6R) abrogate this increase. Next, it was demonstrated that TNFα or IL-6 induce expression of the PD-1Δex3 splice variant in HC CD4+ T cells and that this effect is also abrogated by biologics. It was shown that sPD-1 is produced in higher levels by SFMC as compared to PBMC and that sPD-1 is detected at high levels in the serum and SF of patients with RA and PsA, but not in healthy or OA controls. A cross-sectional analysis further showed that RA and PsA patients treated with TNFα inhibitor (TNFi) therapy had significantly lower sPD-1 in both the serum and SF as compared to patients not treated with TNFi therapy. RA patients treated with MTX therapy were shown to have lower sPD-1 as compared to RA patients not treated with MTX, although the observed difference was not statistically significant. On the contrary, sPD-1 levels were higher in MTX-treated PsA patients as compared to non-treated PsA patients. Mechanistically, it was demonstrated that a recombinant sPD-1fc chimera, used to mimic naturally occurring sPD-1, was able to abrogate PD-1-mediated suppression of proliferation in HC CD4+ T cell-only cultures. Soluble PD-1fc was also found to induce proliferation in HC CD4+:CD14+ co-cultures but had no effect on Treg-mediated suppression of proliferation or cytokine production.

In the literature several pieces of evidence indicate that soluble inhibitory receptors play important roles during immune responses contributing, in some cases, to disease pathology. One example is the leukocyte-associated Ig-like receptor

(LAIR)-1. LAIR-1 is a collagen receptor that inhibits the immune cell function upon collagen binding. The soluble counterpart of LAIR-1, named LAIR-2, has been shown to prevent cross-linking between LAIR-1 and collagens. LAIR-2 can be induced *in vitro* by stimulation of PBMC with PMA and ionomycin and it is increased in the synovial fluid of patients with rheumatoid arthritis as compared with osteoarthritis patients (397). The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another well-characterized inhibitory receptor that exists both in a membrane-bound form and in a soluble form (sCTLA-4) (398-401). Soluble CTLA-4 has been described in human serum and its presence has been associated with several autoimmune diseases including systemic lupus erythematosus and autoimmune thyroid disease (47, 402-405).

Recently, thanks to the extensive studies performed by the scientific community on the PD-1 pathway in cancer immunotherapy, sPD-1 has emerged as an important immunologic modulator both in cancer as well as in autoimmunity. The biological activity of sPD-1 has been studied mainly in antiviral and antitumor immunity while its role in inflammatory arthritis is not well established. In antiviral and antitumor immunity, several authors have shown that sPD-1 is able to interfere with PD-1-mediated inactivation of cytotoxic T cells leading to enhanced T cell ability to fight against cancerous and infected cells (406-409).

He *et al.* used a eukaryotic plasmid (pPD-1A) that expresses the extracellular domain of murine PD-1 (sPD-1) and demonstrated that sPD-1 can bind PD-1 ligands PD-L1 and PD-L2 *in vitro* and enhance anti-tumour immunity *in vivo* (406). Similarly, Shin *et al.* have shown that sPD-1 can synergise with herpes simplex virus thymidine kinase (HSVtk) leading to tumour regression (407). In this study it was shown that in HSVtk expressing tumour cells, exposure to the prodrug ganciclovir

(GCV), leads to inhibition of DNA polymerase, single-strand DNA breaks and tumour cell death by direct cytotoxicity. Soluble PD1 cDNA was integrated into a replication-deficient adenovirus (Ad5mTR.sPD-1) harboring HSVtk and this was used to show that both *in vitro* and *in vivo*, sPD1 synergised with HSVtk boosting antitumor immunity by amplifying CD8+ T cell responses via blockade of PD-L1 in the tumour microenvironment (407). Finally, Sorensen *et al.* compared the concentration of sPD-1 in the serum of advanced epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer patients prior to treatment with EGFR inhibitor drug erlotinib and at the time of progression. Interestingly, sPD-1 was detected in the serum of patients and further increased upon therapy showing an association between sPD-1 levels and prolonged patient survival (410). Further evidence supporting the importance of sPD-1 in T cell regulation can be found in simian antiviral immunity. It has been shown that blockade of PD-1 ligands *in vitro* using a soluble recombinant PD-1fc fusion protein can improve CD4+ and CD8+ antigen-specific T cell responses (408). Administration of sPD-1fc, *in vivo*, enhances simian immunodeficiency virus (SIV)-specific CD4+ and CD8+ T cell proliferation both in the blood and the gut (408). In line with these findings, Onlamoon *et al.* showed that sPD-1 led to increased SIV-specific proliferation of CD4+ and CD8+ T cells in rhesus macaques (409).

In human inflammatory arthritis, the increase in PD-1 expression suggests that this receptor has the potential to reduce inflammation by controlling excessive T cell activation. However, fragmented data exist regarding i) the driving factors of sPD-1 production and ii) whether or not sPD-1 has the ability to interfere with the PD-1:PD-L1 interaction, thereby blocking the negative signal from membrane-bound PD-1.

In regard to sPD-1 production, Nielsen *et al.* have shown that PD-1Δex3 transcript is increased in HC PBMC following TCR activation and CD28 co-stimulation as compared to non-activated cells (63). Furthermore, Wan *et al.* demonstrated that *ex vivo* bulk T cells purified from RA PBMC and SFMC have higher expression of the PD-1Δex3 splice variant compared to healthy and OA controls (324). In line with these findings, Liu *et al.* recently demonstrated that bulk PBMC analysed *ex vivo* from patients with RA have increased PD-1Δex3 transcript as compared to OA and HC PBMC (366). These data suggest that soluble PD-1, the putative translational product of PD-1Δex3, could be produced by both PBMC and SFMC. Furthermore, data also suggest that the increase in the PD-1Δex3 transcript, which is found in RA PB and SF-derived T cells, might be caused by sustained proinflammatory cytokine exposure from the environment in which these cells are found.

The data presented in this chapter support this theory as they demonstrate that *in vitro,* sPD-1 is detected in RA and PsA PBMC culture supernatants, although at very low levels, and it is further increased in SFMC cultures. The data also demonstrate that TNFα or IL-6, which were found to be increased in the arthritic joint, are capable of inducing both PD-1Δex3 splice variant and sPD-1 protein in HC CD4+ T cells and that this cytokine-mediated effect is abrogated by the biologics adalimumab (anti-TNFα) and tocilizumab (anti-IL6R).

Thus far, the PD-1Δex3 splice variant appears to be the only reported and validated mechanism driving the production of sPD-1 and no other mechanisms have been described. Nonetheless, it is possible that a certain amount of PD-1 might be released from the cell membrane via other as yet undescribed mechanisms including shedding of the membrane-bound receptor. An interesting recent report, demonstrated

that expression of the PD-1 ligands (PD-L1 and PD-L2) in infant foreskin fibroblasts can be regulated through proteolytic cleavage by metalloproteinases (MMPs) including MMP-9 and MMP-13 (411).

MMPs such as MMP-9 and MMP-13 have been previously described in the arthritic joint (412, 413) and future studies may reveal whether these MMP molecules might have also a role in the generation of sPD-1.

Importantly, the experiments performed in this chapter on four sets of patient samples (2 RA and 2 PsA) found that sPD-1 can be detected in the serum and synovial fluid of both arthrides but not in healthy and OA controls. These results are important as they confirm that sPD-1 is present during chronic inflammation. These data also support and extend the findings of three recent studies (324, 354, 366), which focused only on RA patients. The studies from Wan *et al.* and Liu *et al*. found that sPD-1 serum levels positively correlated with the presence of rheumatoid factor (RF) and with the levels of TNFα in either the serum or SF of RA patients (324, 366). The results from the two groups are, however, contradictory with one group reporting a positive correlation between sPD-1 and TNFα in the serum (366) while the other reporting a positive correlation between sPD-1 and TNFα only in the synovial fluid but not in the serum (324). Data from this thesis confirm that sPD-1 and TNFα positively correlate in the RA serum while no significant correlation between other cytokines and either sPD-1 levels, Disease Activity Scores (DAS28), Tender Joint Count (TJC) and Swollen Joint Count (SJC) are found. Such differences might be attributed to many factors including the different cohorts selected for the studies as well as the different treatments and numbers of the recruited patients.

In this chapter, the role of methotrexate (MTX) and TNFα inhibitor (TNFi) therapies in modulating sPD-1 levels was also investigated. This is of interest because high sPD-1 levels in both serum and SF as well as variations in response to therapy could potentially be used to evaluate disease activity. Greisen *et al.* (354) showed that in a longitudinal set of plasma samples from the CIMESTRA study with early RA patients evaluated at 0 and 9 months after MTX therapy, plasma levels of sPD-1 decreased after 9 months and were comparable to the levels detected in healthy volunteers. Soluble PD-1 was found to strongly correlate with IL-21, IgMRF and anti-CCP antibodies (354) indicating a possible role in disease progression. Interestingly the same group also showed that in a cross-sectional analysis of plasma and synovial fluid (SF) from patients with chronic RA, sPD-1 was higher in the SF than in the plasma suggesting also an important role for this molecule when the disease is well established and present for years.

In line with longitudinal analysis from previous reports (354, 366), the cross-sectional analysis performed in this thesis on RA and PsA serum and SF revealed that RA patients treated with methotrexate (MTX) therapy had overall lower sPD-1 levels in both serum and SF compared to RA patients not treated with MTX therapy, although the observed differences were not statistically significant. Interestingly, this was not the case for PsA where MTX-treated patients had significantly higher levels of sPD-1 in both serum and SF as compared to patients not treated with MTX therapy. These data suggest that in RA and PsA patients, MTX therapy might modulate sPD-1 levels in yet undescribed mechanisms. The cross-sectional analysis performed in this thesis further revealed that RA and PsA patients treated with TNFi therapy had lower levels of sPD-1 both in the serum and SF as compared to patients not treated with TNFi therapy. Although the observed differences were greater in RA than in PsA,

these results are in line with *in vitro* data (chapter 5, section 5.2.1) showing that anti-TNFα biologic adalimumab is able to reduce PD-1Δex3 transcript and sPD-1 production in HC CD4+ T cell cultures. Furthermore, they also partially align with our preliminary longitudinal analysis showing that sPD-1 serum levels decreased overtime upon TNFi therapy in 2 of 4 samples tested.

The role of therapeutics in modulating sPD-1 in RA and PsA serum and SF is of clinical interest. However, it is important to point out that the observed differences between RA and PsA described in this chapter might be a consequence of the cross-sectional nature of the analysis performed as well as the limited number of patient samples, which were available at the time when this work was performed. Hence, further longitudinal studies on RA and PsA patients treated with MTX, adalimumab or tocilizumab are required before conclusive statements can be made regarding the effect of therapy *in vivo*.

As mentioned above, the modulatory role of sPD-1 has been predominantly investigated in murine antitumor and simian antiviral immunity while the role of sPD-1 during PD-1:PD-L1 interactions in human T cells is not well established. Wan *et al*. showed that PD-1 fusion protein (PD-1fc) added to isolated RA synovial fluid CD4+ T cells cultured with irradiated autologous SFMC is able to induce cell proliferation in a dose-dependent fashion (324). Similarly, Liu *et al*. cultured RA CD4+ T cells with a stable human PD-L1 transfected cell line (L929-PD-L1) and described an increase in cell proliferation upon treatment with PD-1fc (366). Although these results suggest that sPD-1 might induce proliferation in RA CD4+ T cells co-cultured with other cell types they did not directly prove that sPD-1 is able to abrogate PD-1 ligation with its ligand PD-L1.

Hence, further experiments performed in this thesis attempted to assess whether sPD-1 displayed functional antagonism in HC CD4+ T cell and could inhibit membrane-bound PD-1 ligation by its ligand PD-L1. Data presented in this chapter demonstrated that sPD-1 is biologically active *in vitro* and can counteract PD-1-mediated suppression of proliferation of healthy CD4+ T cell in a dose-dependent fashion (section 5.2.3, Figure 5.12). Furthermore, data also showed that sPD-1 enhances HC CD4+ T cell proliferation when co-cultured with autologous CD14+ monocytes, which naturally express PD-L1 and further upregulate it in presence of IFN-γ, IL-6 or TNFα (section 5.2.3, Figure 5.14). These results are important as they provide evidence for the biological role of sPD-1 and further suggest that during chronic inflammation sPD-1 might interfere with the PD-1 pathway thereby disrupting T cell regulation. This is supported by two recent human studies. In the first study, Aarslev *et al.* showed that in autoimmune hepatitis (AIH) sPD-1 is significantly elevated in AIH patients with active disease and in incomplete responders to standard therapy compared with responders and healthy controls (414). In the second study, Yanaba *et al*. described that patients with diffuse cutaneous systemic sclerosis (SSc) have higher levels of sPD-1 than those with limited SSc or healthy individuals and that sPD-1 levels in the serum of patients positively correlate with the severity of skin disease (415).

Finally, the effect of sPD-1 was investigated in T regulatory (Treg) cell-mediated suppression. Treg cells and the PD-1 pathway are both critical in controlling immune responses. Elimination of either one can lead to strong T cell responses and development of autoimmunity (98, 394). CD4+CD25+ Treg cells are important in controlling the severity of arthritis and mouse studies have shown that exacerbated

disease can be detected following Treg cells depletion (416, 417) while CD4+CD25+ adoptive transfer leads to reduction in disease duration (418). Treg cells are increased in the inflamed joints of patients with RA and PsA (334, 338-343, 419, 420) but despite their presence, inflammation persists, and it is unclear whether these cells are impaired in different arthrides.

Functionally, some groups have reported that Treg cells from the peripheral blood of RA patients are unable to suppress T effector (Teff) cell proliferation (272, 421, 422) while the majority of studies, however, have shown that peripheral blood CD4+CD25+ Treg cells have an intact regulatory function and they are capable of regulating the proliferation of Teff cells *ex vivo* (325, 339, 342, 344, 423-425). Similarly, CD4+CD25+ Treg cells from the inflamed joints of patients with arthritis have also been shown to be functionally intact and capable of suppressing proliferation and cytokine production *ex vivo* (338-344, 419, 426, 427).

Notably, the PD-1:PD-L1 interaction has been shown to be important for the maintenance of a correct Treg function. Francisco *et al.* demonstrated that stimulation with PD-L1fc-coated beads can increase the *de-novo* generation of CD4+FoxP3+ Tregs from naïve CD4+ T cells. PD-L1fc was also shown to enhance both FoxP3 expression and the suppressive function of established induced Tregs (iTregs). Mechanistically, it was demonstrated that PD-L1fc induced iTreg cells from naïve T cells by downregulation of the Akt-mTOR signalling and upregulation of PTEN (98, 394). This is in line with studies demonstrating the effects of rapamycin and mTOR inhibition on the development of Treg cells (428-430).

These findings suggest that any disruption of this pathway could potentially have negative effects on Treg cells functions. For example, an antagonist effect mediated by sPD-1, which in this thesis was shown to be highly upregulated in RA

and PsA, could partially block PD-1:PD-L1 interaction modulating the maintenance of regulatory T cell function within the joints of RA and PsA patients.

To further investigate this possibility, a Treg:Teff suppression assay was set up and the effect of sPD-1 on Treg-mediated suppression was evaluated. Initial experiments confirmed that *in vitro*, CD4+CD25+ Treg cells from healthy volunteers were able to negatively modulate autologous CD4+ and CD8+ T cell proliferation and to modulate the percentage of PD-1+ cells in the co-cultures. Unexpectedly, addition of sPD-1fc to Teff cells in absence of Treg cells did not enhanced proliferation. Furthermore, addition of sPD-1 to Treg:Teff co-cultures did not modulate Treg cells-mediated suppression of proliferation nor cytokine production. These results are partially in contrast with previous data showing that sPD-1 is able to induce cell proliferation in HC CD4+ T cells cultured with autologous CD14+ monocytes. In the selected suppression assay, bulk PBMC were used as effectors in line with a previous investigation (431) and with the intent to recreate a more physiological environment without eliminating different accessory cells normally found during immune responses *in vivo*. The absence of sPD-1-mediated effects might be dependent on the experimental design used in this specific set of experiments. Addition of sPD-1 to bulk PBMC might lead to activation of yet undiscovered biological mechanisms, which might be in place to prevent cell proliferation. This could be investigated further by re-designing the experiments and culturing Treg cells in presence of isolated CD4+ or CD8+ T cells as effectors and with autologous CD14+ monocytes as APC to mimic the CD4+:CD14+ set up of our previous experiments. The different sensitivity of the methods used to detect cell proliferation (CTV in the Treg-suppression assay versus [^{3}H]-thymidine uptake in CD4+:CD14+ co-cultures) might also be partially responsible for the observed results. CTV dye dilution was chosen for

this specific test because of our interest in using flow cytometry to have a more exhaustive and comprehensive analysis of the different cell populations involved in the assay. Additional tests using [^{3}H]-thymidine incorporation might reveal whether the observed results are due to a sensitivity issue. Finally, the loss of biological activity of the soluble PD-1fc chimera and the variability of the donors tested might have also played a role. Further experiments with increased numbers of donor as well as with new batches of reagents might generate a different outcome and help to clarify better if sPD-1 has a role in modulating Treg-mediated suppression.

In conclusion, this chapter provides additional evidence for sPD-1 as a putative modulator of PD-1-mediated regulation in human CD4+ T cells and suggest that in both RA and PsA, sPD-1 might have a role in disease progression.

6 General Discussion

In this final chapter the key findings presented in this thesis will be summarised, followed by a discussion of important outstanding questions related to this work and potential directions for future work.

6.1 Summary of results described in this thesis

The work presented in this thesis was designed with the intent to (i) bring together individual pieces of data in the disease context of RA, (ii) extend them into a different inflammatory joint disease to validate the general principle of impaired PD-1-mediated regulation, and (iii) investigate why such a prominent regulatory pathway is not working effectively in the context of inflammatory arthritis. To address this, I have provided data showing that in RA as well as in PsA patients the PD-1 receptor is upregulated on synovial T cells and that CD4+ T cells from PB and SF of either patient group are resistant to PD-1 ligation *in vitro*. Next, I showed that proinflammatory cytokines TNFα, IL-6 and IL-1β are increased in both RA and PsA and they act as negative modulators of PD-1 function *in vitro.* In addition, I demonstrated that current biologics (adalimumab and tocilizumab) used routinely in patient treatment, as well as anti-IL-1β, are able to counteract this cytokine-mediated effect. These data add novel insight to the field of rheumatology and demonstrate that inflammatory cytokines typically associated with arthritis (TNFα, IL-6 and IL-1β) have a role in counteracting the suppressive effect of PD-1 ligation on CD4+ T cells. Results discussed here also indicate that T cell and monocyte-derived inflammatory cytokines have a profound effect on PD-1-mediated T cell regulation. My data show that biologic drugs directly abrogate this cytokine-mediated effect and successfully

restore PD-1-mediated regulation of T cell proliferation. Finally, I confirmed that TNFα drives soluble PD-1 production in HC CD4+ T cells and I described that IL-6 has also such an ability, which has not been shown previously. In addition, I showed that sPD-1 levels are lower in RA and PsA patients undergoing TNFi therapy and in RA patients undergoing MTX therapy as compared to patients not treated with anti-TNF or MTX. Together, these findings form an informative data set that advances our knowledge on the function of the PD-1/PD-L1 signalling pathway in inflammatory arthritis. Furthermore, this is the first study to examine PD-1 function in RA and PsA simultaneously, and to provide evidence that the inflammatory milieu of these two diseases has a role in negatively modulating PD-1 ligation.

In chapter 3 the analysis of a gene expression array performed on RA PB- and RA SF-derived cells demonstrated that the expression of PD-1 is increased in SF Teff and Treg cells as compared to PB cells. In line with these data, flow cytometry analysis also demonstrated that the frequencies of PD-1+ cells within CD4+ and CD8+ T cell subsets and in CD4+CD25+ Treg cells are increased significantly in the SF from patients with RA or PsA when compared to PB, thus extending the findings from previous studies focusing only on RA-derived CD4+ Teff cells (216, 323, 324, 432). The observed increase in the frequencies of PD-1+ T cells suggests that in RA and PsA, PD-1 might have a role in regulating Teff and Treg cells, especially at the site of inflammation. Interestingly, despite increased frequencies of PD-1+ T cells, inflammation persists, further suggesting that this pathway is impaired in these diseases. Lower percentages of PD-L1+CD14+ cells were found in the SF of RA and PsA patients as compared to PB.

This is line with a previous report further showing that similar expression levels of PD-L1 can be found between RA and OA-derived synovial tissue and suggesting that synovial PD-L1 concentrations, in certain groups of patients, might not be adequate to effectively downregulate autoreactive T cells (216)

PD-1 function in inflammatory arthritis is still widely unexplored. One study reported that PD-1 ligation inhibited cell proliferation and IFN-γ production by CD4+ T cells from peripheral blood of RA patients. The same study further demonstrated that synovial fluid CD4+ T cells required higher concentrations of PD-L1fc to achieve similar levels of inhibition (216). The authors speculated that the inflammatory milieu found in the RA joint might be accountable for reduced PD-1-mediated suppression. However, no specific mediator of the effect was identified (216).

It has been shown previously that the common gamma chain cytokines IL-2, IL-7 and IL-15 as well as CD28 co-stimulation, can interfere with PD-1 crosslinking (60, 368). Hence, in chapter 4, I sought to investigate i) the ability of PD-1 ligation to regulate proliferation and cytokine production in RA- and PsA-derived CD4+ T cells and ii) the role of inflammation as a possible negative modulator of PD-1-mediated regulation. My data demonstrated that CD4+ T cells from both blood and synovial fluid of RA and PsA patients are more resistant to PD-1 ligation in terms of suppression of T cell proliferation and IFN-γ production when compared to healthy cells. Data also demonstrated that proinflammatory cytokines TNFα, IL-6 and IL-1β abrogate PD-1-mediated suppression of proliferation in healthy CD4+ T cells and that cytokines inhibitors counteract these effects *in vitro.* In line with these data, I was able to show that cell-free SF from RA or PsA patients added to healthy CD4+ T cell cultures was also able to reduce PD-1-mediated suppression of proliferation. These

findings are important as they provide new evidence that the presence of certain proinflammatory cytokines typically found in RA and PsA can be critical in determining the outcome of PD-1 engagement during the immune response.

The aim of the experiments described in chapter 5 was to identify and test a mechanism, which would explain, at least in part, how inflammation modulates PD-1-mediated regulation. For this purpose, cell supernatants from HC CD4+ T cell cultures stimulated with proinflammatory cytokines were analysed and it was found that TNFα and IL-6, but not IL-1β, induced the secretion of sPD-1. Importantly, *ex vivo* analysis confirmed that sPD-1 levels were significantly increased in the serum and SF of patients with RA or PsA compared to healthy or OA controls pointing to a biological role of sPD-1 both systemically and locally in the inflamed joint of different inflammatory arthrides. The latter data support and extend two recent studies showing that sPD-1 is detected in the serum and SF of patients with RA (324, 354).

Recent studies have also shown that expression of the PD-1Δex3 splice variant is observed in T cells from patients with RA, but only minimally in T cells from patients with OA or from HC (324, 366). In healthy human CD4+ T cells it appears that cytokines TNFα, IL-17 and IFN-γ drive the expression of PD-1Δex3 mRNA (366). My data confirmed that in HC CD4+ T cells, TNFα drives PD-1Δex3 splice variant, and further demonstrated that PD-1Δex3 splice variant and sPD-1 protein are regulated by IL-6 and can be negatively modulated by therapeutic antibodies adalimumab and tocilizumab.

Preliminary analysis from a cross-sectional investigation of RA and PsA serum and SF also showed that patients treated with TNFi therapy have lower sPD-1 levels compared to patients not treated with TNFi therapy and that this is also the case for RA patients treated with MTX therapy as compared to patients not treated with MTX. TNFi therapy data are novel and in line with my experiments showing that anti-TNFα (adalimumab) is able to reduce both PD-1Δex3 splice variant mRNA and sPD-1 protein *in vitro*. MTX data are also novel and in line with a longitudinal analysis performed by Liu *et al.*, which measured serum levels of sPD-1 in RA patients before and after treatment with MTX and reported a decrease following MTX medication (366).

Data from chapter 5 indicate that certain cytokines can induce PD-1Δex3 and sPD-1 production. However, metalloproteinases (MMPs) such as MMP-9 and MMP-13, which have been previously described in inflammatory arthritis (412, 413), might also be responsible for the generation of sPD-1 through proteolytic cleavage. This is supported by a recent investigation showing that expression of PD-L1 and PD-L2 is regulated by MMPs in infant foreskin fibroblasts (411). Notably, the experiments performed in chapter 5 have also shown that a recombinant PD-1 chimera (sPD-1fc), used to mimic naturally occurring sPD-1, is functionally able to counteract PD-L1fc-mediated suppression of healthy CD4+ T cell proliferation. Soluble PD-1fc was also found to enhance CD4+ T cell proliferation when HC CD4+ T cells are co-cultured with autologous CD14+ monocytes that can naturally express PD-L1. These findings indicate that sPD-1 might act as a decoy during APC-mediated regulation of T cells via PD-1/PD-L1 signalling.

Based on previous literature discussed in chapter 5, which suggests that disruption of PD-1:PD-L1 interactions negatively modulates Treg cell function, this thesis investigated whether sPD-1 negatively modulated Treg function *in vitro* using a Treg suppression assay. Soluble PD-1 had no effect on Treg-mediated regulation of proliferation nor on cytokine production and further experiments are required before conclusive statements can be made regarding the effect of sPD-1 in a Teff:Treg co-culture system. However, recent evidence from studies performed in inflammatory diseases such as chronic autoimmune hepatitis (AIH) (414) and cutaneous systemic sclerosis (SSc) (415) strongly support the notion that sPD-1 might actively interfere with the PD-1 pathway, thereby disrupting T cell regulation (see chapter 5 discussion). Importantly, and in line with data presented in this thesis, cytokines TNFα and IL-6, two inducers of PD-1Δex3 splice variant (380), can be detected in AIH (433) and in SSc (434, 435) supporting the hypothesis that sPD-1 is a product of the inflammatory milieu and providing further evidence of a bioactive function of this soluble molecule.

The bioactive function of sPD-1 is also evident in cancer. It is now widely demonstrated that PD-L1 positive tumour cells engage with PD-1 positive tumour-infiltrating T cells to downregulate their cytotoxic functions (207). Soluble PD-1 is detected in the tumour microenvironment where it engages with PD-L1-expressing tumour cells disrupting their evasion mechanism and promoting T cell cytotoxicity. In a murine model of hepatocarcinoma it was shown that combined treatment of IL-21 and sPD-1 enhanced T cell cytotoxicity, increased the number of cytotoxic T cells and NK cells and upregulated the cytokines IFN-γ and IL-2 resulting in a more efficacious antitumor immune response (436). Bartee *et al.* generated a recombinant myxoma virus (vPD-1), which inhibits the PD-1/PD-L1 pathway by secreting a soluble form of

PD-1 from infected cells, and demonstrated that tumour-localized secretion of sPD-1 strongly modulates anti-tumour CD8+ T cell responses (437). These studies are in line with reports by He *et al.* (406) and Shin *et al.* (407) in mice, and work by Amancha *et al.* (408) and Onlamoon *et al*. (409) in simians (see chapter 5 discussion).

The mechanisms leading to sPD-1 in the tumour microenvironment are currently unclear. Inflammatory mediators such as TNFα, IL-6, IFN-γ, IL-17, TGF-β, and IL-10 (438, 439) and metalloproteinases MMP-9 and MMP-13 (440) are found in the tumour microenvironment and different cytokines have been shown to participate in both the initiation and progression of cancer through different mechanisms including upregulation of PD-L1 on tumour cells (207, 438, 439). However, it remains to be determined whether cytokines such TNFα and IL-6 are also able to exert anti-tumorigenic effects through direct induction of sPD-1 and whether MMP-9 and MMP-13 can cleave membrane-bound PD-1 and its ligands in the tumour microenvironment.

Interestingly, PD-L1 is also present in some cancer patients in a soluble form (sPD-L1) and it has been associated with immune suppression and poor prognosis in patients with advanced lung cancer (441), renal cell carcinoma (442), large B cell lymphoma (443), nasal natural killer/T-cell lymphoma (444), multiple myeloma and melanoma (445).

This suggests that sPD-1 and sPD-L1 are regulatory factors to the PD-1/PD-L1 signalling pathway, each having opposite effects. In inflammatory arthritis limited data is available on sPD-L1. sPD-L1 appears to be upregulated in the serum and synovial fluid of RA patients and serum levels correlate with rheumatic factor (RF) (324). However, it is not known whether sPD-L1 has a downregulatory function and can engage with membrane-bound PD-1 blocking T cell activation and cytokine

production or if it binds to sPD-1 leading to formation of sPD-1:sPD-L1 complexes at the site of inflammation.

Collectively, data presented in this thesis provide new evidence that the inflammatory environment of the RA and PsA joint compromises PD-1/PD-L1-mediated T cell regulation and that production of sPD-1 might counteract the function of overexpressed PD-1 to restrict inflammation.

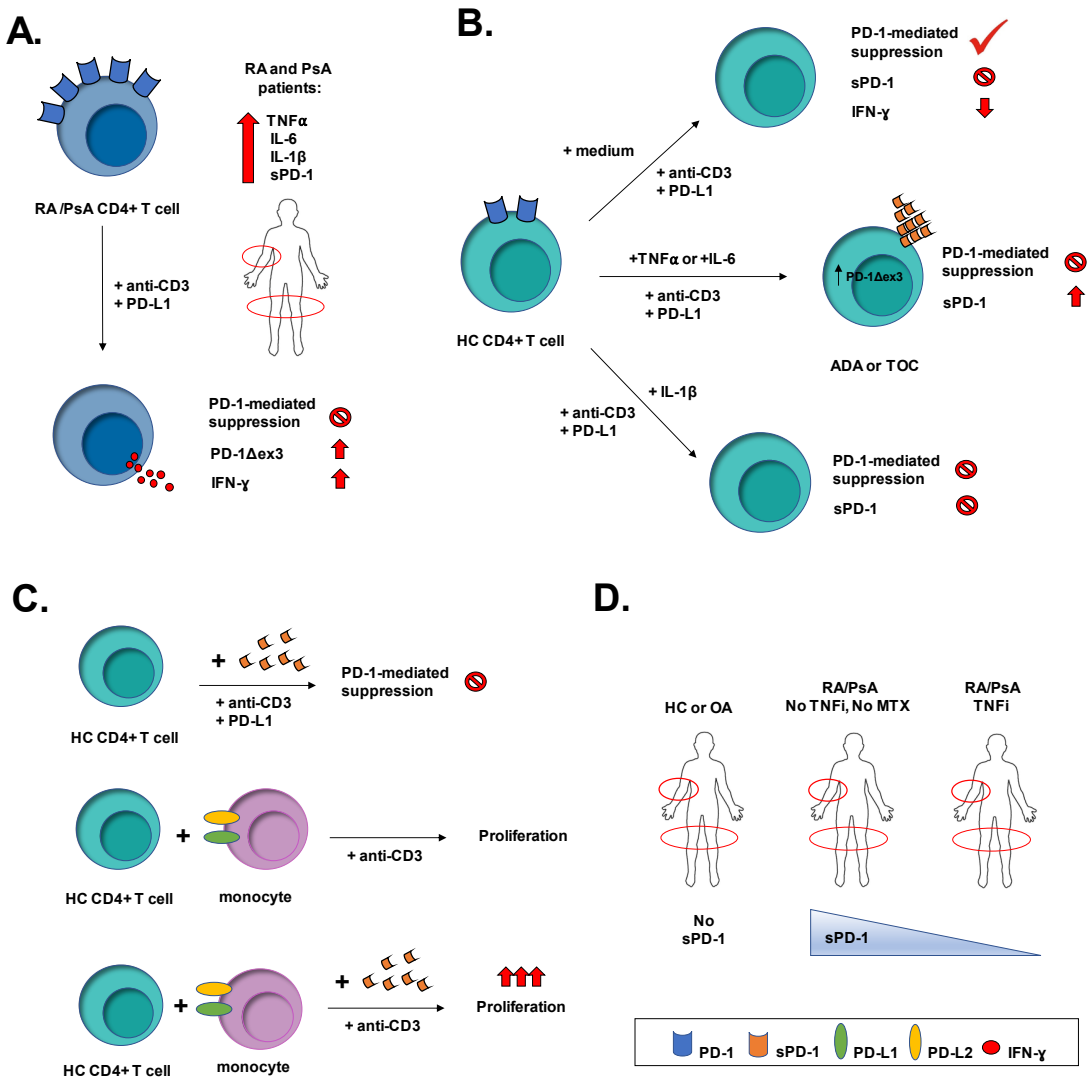

Figure 6.1 Schematic overview of selected findings from this thesis.
(A) RA and PsA PB and SF-derived CD4+ T cells cultured in the presence of anti-CD3 mAb and PD-L1 chimera are resistant to PD-1-mediated suppression of proliferation and cytokine production. RA-derived cells express PD-1Δex3 (324). Increased levels of TNFα, IL-6 and IL-1β are detected in both RA and PsA SF when compared to HC and OA controls. (B) The presence of TNFα, IL-6 or IL-1β is able to abrogate the suppressive effects of PD-1 ligation on healthy CD4+ T cells suggesting that inflammation negatively modulates PD-1-mediated regulation. Soluble PD-1 is detected in the supernatants of TNFα- and IL-6-stimulated cultures and HC CD4+ T cells from the same cultures show increased expression of the PD-1Δex3 splice variant. (C) Soluble PD-1 modulates PD-1-mediated suppression of HC CD4+ T cell proliferation and induces proliferation in CD4+T cell:CD14+ monocyte co-cultures. (D) sPD-1 is not detected in HC and OA controls, while it can be found at high levels in RA and PsA serum and SF. Furthermore, sPD-1 levels are lower in RA and PsA patients undergoing TNFi therapy as compared to patients not treated with TNFi or MTX therapy.

6.2 Outstanding questions and future investigation

This part of the discussion analyses new lines of research that could be pursued to implement the data already generated in this thesis and to extend our knowledge about T cell regulation in inflammatory arthritis. In the next sections I will describe possible new experiments on three different topics including:

- The role of PD-L2 (B7-DC) in RA and PsA
- The role of PD-1 in modulating Treg cell-mediated suppression
- The role of soluble CD80 (sCD80) in T cell regulation

6.2.1 Does PD-L2 modulate T cell proliferation of RA- and PsA-derived CD4+ T cells and in CD4+:CD14+ co-cultures?

The work presented in this thesis has shown that CD4+ T cells derived from RA and PsA patients are resistant to PD-1-mediated regulation when cultured in presence of PD-L1 *in vitro*. However, as discussed in chapter 1, PD-1 has a second ligand named PD-L2 (61, 62), which has a three-fold higher affinity for PD-1 as compared to PD-L1 and is able to inhibit T cell proliferation and cytokine production (61). PD-L2 is also expressed on activated human CD4+ and CD8+ T cells and PD-L2 engagement at the surface of T cells appears to reduce cytokine production and cell proliferation (446). Several lines of evidence suggest that PD-L2 is important to prevent autoimmunity. PD-L2$^{-/-}$ mice have increased activation of CD4+ and CD8+ T cells *in vivo* when compared to wild type (WT) mice and lack of PD-L2 has been shown to modulate T cell tolerance (447). Following PD-L2 blockade, exacerbation of disease is observed in mouse models of experimental autoimmune encephalomyelitis (EAE) (213) and autoimmune kidney disease (448) while PD-L2 polymorphisms are associated with susceptibility to systemic lupus erythematosus (SLE) in humans (449).

In RA and PsA very little is known about PD-L2. Recently, Greisen *et al.* showed that PD-L2 knockout mice have increased bone mass density compared to wild type mice and that PD-L2 can be detected in pre-osteoclasts suggesting a role of PD-L2 in bone homeostasis (450). PD-L2 expression is increased in RA synovial tissue as compared to healthy tissue but PD-L2 levels are similar between RA and OA (216). PD-L2 expression pattern in PsA has never been investigated and whether PD-L2 is able to control proliferation and cytokine production in RA- and PsA-derived T cells have never been tested either. Hence, further experiments designed to test PD-L2 suppressive activity in T cells are of clinical interest. To answer this, different research approaches could be pursued.

Firstly, CD4+ and CD8+ T cells from the blood of healthy volunteers and from the blood and SF of RA and PsA patients could be cultured *in vitro* in presence of increasing doses of PD-L2fc ligand. My hypothesis is that these experiments will confirm that RA- and PsA-derived cells are resistant to PD-1 ligation even in presence of PD-1 second ligand PD-L2. These experiments would be repeated in presence of both PD-L1fc and PD-L2fc ligands to address whether the two inhibitory ligands have a joint effect on healthy- and patient-derived cells. Similarly to experiments performed in this thesis using PD-L1fc, my hypothesis is that TNFα, IL-6, IL-1β or sPD-1 addition to the cell culture will abrogate PD-1-mediated suppression of proliferation in healthy CD4+ T cells cultured in presence of PD-L2fc by a sPD-1-dependend mechanism as seen for PD-L1fc.

Data from this thesis also demonstrated that addition of exogenous sPD-1 to HC CD4+:CD14+ co-cultures modulates T cell proliferation in a dose-dependent fashion (380). As CD4+ T cells alone did not proliferate following sPD-1 stimulation, this indicates that, *in vitro,* sPD-1 likely interferes with PD-L1 and possibly PD-L2

expressed on monocytes. One question that arose from my CD4+:CD14+ co-culture experiments and that has not been addressed is whether sPD-1 is able to interfere with PD-L1, PD-L2 or both ligands. This is of interest because it would provide new information about sPD-1 modulatory activity.

To address this point the CRISPR-Cas9 genome editing technique could be used to generate PD-L1 knockout (KO), PD-L2 KO and PD-L1/PD-L2 KO monocyte-derived dendritic cells. Generally it takes several days to perform CRISPR-Cas9 and the technique typically requires selection to enrich for Cas9 transfected cells. Since monocytes have a short lifespan in culture, it would not be possible to perform gene editing fast enough to allow time for functional assays. A possible solution for this issue could be to use already differentiated APCs or to target a progenitor cell type and then differentiate monocytes *in vitro* from a genome-edited precursor.

Following genome editing, PD-L1/2 KO cells would be incubated with autologous CD4+ T cells and cultured with anti-CD3 and increasing concentrations of sPD-1. My hypothesis is that if sPD-1 binds specifically to PD-L1, no increase in proliferation should occur in the PD-L1-KO condition. Conversely if sPD-1 binds only to PD-L2, no increase in proliferation should occur in the PD-L2-KO condition. This experiment would identify whether sPD-1 binds to both PD-L1 and PD-L2 ligands or only to one of them. In a different approach CD14+ monocytes could be pre-treated with blocking antibodies towards either PD-L1 or PD-L2 to modulate their ability to crosslink PD-1. Next, treated monocytes would be incubated with autologous CD4+ T cells and cultured with anti-CD3 and increasing concentrations of sPD-1. One limitation of this experiment lays in the fact that it is unclear whether blocking antibodies for PD-L1 and PD-L2 induce reverse signalling in the monocytes.

Hence, this should be accurately tested during the experiments, for example, by analyzing cytokine production and cell signalling molecules.

6.2.2 Does PD-1 modulate Treg-mediated suppression in RA and PsA?

A key outstanding question that I have begun to address in this thesis is whether sPD-1 regulates the suppressive function of Treg cells *in vitro*. Soluble PD-1 levels are high in the serum and SF of RA and PsA patients (chapter 4) and whether sPD-1 has an effect on Treg cell-mediated suppression is currently unknown. The immune-inhibitory PD-1 receptor is expressed on activated T cells, including FoxP3+ Treg cells. However, it is unclear whether PD-1 expression on Treg cells is required for their immune regulatory function, especially in autoimmune settings. Francisco *et al.* demonstrated that in the presence of anti-CD3 and TGF-β, PD-L1-Ig induces a profound increase in *de novo* generated CD4+FoxP3+ Tregs from naïve CD4+ T cells while PD-L1$^{-/-}$ APCs are unable to properly convert naïve CD4+ T cells to iTreg cells. PD-L1-Ig was also shown to enhance FoxP3 expression and suppressive functions of established iTregs suggesting an essential role of PD-L1 for iTreg cell induction and function (98). More recently, Zhang *et al.* (451) described that in mice, deficiency of PD-1 affects the Treg compartment by increasing the generation of ex-FoxP3 cells (452) (T helper cells derived from FoxP3+ T cells) and contributing to the expansion of effector/memory T cells suggesting that PD-1 regulates the relative abundance of Treg and effector T cells to maintain immune tolerance (451). Because depletion of CD25+CD4+ Treg cells or ablation of FoxP3+ Treg cells leads to development of autoimmunity (453-455), I began to test the hypothesis that sPD-1 can interfere with PD-1:PD-L1 interactions and have a negative impact towards the suppressive function of Treg cells.

In my experiments whole PBMC were used as effector cells. This choice was made with the intent to recreate a more physiological experimental condition and keeping in account that different cell populations, such as T cells, B cells and APCs express PD-L1 upon activation and might use it to crosslink PD-1 on Treg cells modulating their ability to suppress proliferation and cytokine production. However, the data obtained from the Treg suppression assay showed that sPD-1 added from the beginning of the co-culture did not modulate Treg cells ability to control proliferation and cytokine production (chapter 5). Hence, further investigation is needed to test whether PD-L1 on T effector cells is important for Treg cells suppressive functions.

To evaluate the effect of blocking the engagement of PD-1+ Treg cells by PD-L1/2+ Teff cells two different approaches could be tested. In the proposed experiments I would use CD4+CD25+ Treg cells, CD4+CD25- Teff cells and CD14+ monocytes or CD3/CD28 coated beads to provide co-stimulation

In my first approach, CD4+CD25- Teff cells and CD14+ monocytes would be incubated overnight in presence of anti-CD3 stimulation and in absence of Treg cells. This step will initiate upregulation of PD-L1 and PD-L2 on Teff cells and monocytes. After 12 hours, autologous Treg cells would be added for the remaining 36 hrs of culture. Blocking antibodies towards PD-L1 and PD-L2 or increasing concentrations of soluble PD-1 and the appropriate controls would also be added for the remaining 36 hrs of culture. My hypothesis is that PD-L1 blocking antibodies and sPD-1 will abrogate PD-1:PD-L1 interactions having a negative impact towards the suppressive function of Treg cells and resulting in higher Teff cells proliferation as compared to the controls. These experiments should also be performed blocking only one ligand at the time to evaluate whether differences exist between PD-L1 and PD-L2 modulatory activity.

The second experimental approach to evaluate the role of PD-L1 and PD-L2 in this system would involve knocking down PD-L1, PD-L2 or both by CRISPR-Cas9 genome editing in CD4+CD25- Teff cells. PD-L1, PD-L2 or PD-L1/2 knockout Teff cells or control Teff cells would be cultured from the start in presence of CD4+CD25+ Treg cells and stimulated with anti-CD3/CD28 coated beads for 48 hrs. My hypothesis for these experiments is that higher proliferation and higher cytokine production should be detected using Teff cells lacking PD-1 ligands. In these experiments anti-CD3/CD28 beads stimulation could be substituted with addition of CD14+ monocytes to the culture provided that PD-1 ligands are also knocked out or blocked by specific antibodies in these cells. As previously stated in section 6.2.1, CRISPR-Cas9 in primary CD4+ T cells present technical challenges including optimisation of lentiviral transduction, subsequent selection and expansion of clones in which gene editing has occurred successfully and maintenance of cell viability to successfully isolate single cell clones.

6.2.3 Does soluble CD80 (sCD80) pair with sPD-1 to promote persistent T cell activation?

A third topic that requires further investigation is whether soluble CD80 (sCD80) act synergistically with sPD-1 to negatively modulate T cell regulation. The biological activity of the costimulatory molecule CD28 has been extensively studied. CD28 is constitutively expressed by T cells and interacts with the B7 molecules CD80 and CD86 expressed mainly by APCs. This interaction results in increased T cell proliferation, IL-2 production, and resistance to apoptosis (24) while blockade of it via the humanised fusion protein CTLA-4-Ig (abatacept) has shown clear benefits in

patients with different inflammatory arthrides including RA, PsA and JIA (313, 318). Several pieces of evidence suggest that soluble CD80 (sCD80) may have a role in negatively modulating T cell regulation in a similar fashion as sPD-1. Soluble CD80 is increased in the serum and SF of RA patients compared to healthy and OA controls and it positively correlates with the levels of RF (324, 456). CD80 binds to PD-L1 with a binding affinity, which is approximately equal to its affinity for CD28. Importantly, PD-L1:CD80 interactions have been shown to directly downregulate T cell responses *in vitro* (127). This suggests that in RA and possibly in PsA, sCD80 may negatively modulate T cell regulation through three distinct mechanisms: i) binding PD-L1 and preventing PD-L1+ cells from regulating PD-1+ activated T cells (similarly to sPD-1), ii) binding PD-L1 and preventing PD-L1+ cells from regulating CD80+ T cells by reverse signalling through T cell–expressed CD80, and iii) enhancing T cell activation by costimulating T cells through CD28. The hypothesis that sCD80 might be important for the regulation of T cell function is supported by recent studies with human and mouse tumour cells where sCD80 (CD80-fc consisting of the extracellular CD80 IgV and IgC regions fused to an IgG-fc domain) and membrane-bound CD80 prevented PD-L1+ tumour cells from binding to PD-1+ T cells and sustaining IFNγ production by activated PD-1+CD4+ and PD-1+CD8+ T cells (457, 458). In order to investigate the role of sCD80 in inflammatory arthritis, future experiments could be designed as follows: Firstly, the presence of sCD80 should be assessed by ELISA in healthy serum and serum and SF from patients with rheumatoid and psoriatic arthritis. Ideally, the analysis should include tests on newly diagnosed patients and follow-ups after initiation of TNFi and MTX therapy to assess whether sCD80 levels are modulated by treatment overtime and whether different treatments specifically modulate sCD80 levels. Next, sCD80 gene expression analysis

should be performed on *ex vivo* PB- and SF-derived T cells from both RA and PsA and coupled with experiments aimed at detecting sCD80 in cell supernatants before and after cell activation and culture with proinflammatory cytokine and specific blocking antibodies. The rationale behind these experiments is based on data showing that, like sPD-1, sCD80 is generated in human cells by alternative splicing (459) while it is unclear whether specific cytokines drive the transcription of the spliced variant. Finally, *in vitro* functional experiments using sCD80 alone and together with sPD-1 could be performed to investigate whether these soluble receptors are able to modulate T cell proliferation and cytokine production in presence or absence of PD-L1 and PD-L2 ligands. In order to provide a truly comprehensive analysis, these experiments should be performed on PB and PB- and SF-derived CD4+ and CD8+ T cells from healthy volunteers and from RA and PsA patients. These experiments might provide novel data on how T cells are regulated at the site of inflammation in different human arthrides.

6.3 Concluding remarks

The PD-1 receptor is a potent immunoregulatory molecule, which plays an important role in the regulation of T cell activation and in the maintenance of cell tolerance. The onset of autoimmune-like adverse effect in cancer patients treated with anti-PD-1 therapy implies that the converse strategy of engaging the PD-1 receptor to deliver an inhibitory signal might be a promising way to control inflammation in different autoimmune conditions. Multiple approaches are under investigation to target immune checkpoints for the treatment of autoimmune diseases. These new approaches include ligand–fc fusion proteins and artificial ligands. Preclinical studies in the CIA murine model of RA support the feasibility of this therapeutic strategy as they show that

injection of the artificial ligand PD-L1fc (215, 216), CD200fc (460) and PECAM1-derived peptide (461) ameliorate the disease and reduces inflammation. Another therapeutic strategy that is currently under investigation is the use of antibodies called “bispecific” which co-ligate an inhibitory and activating receptor enhancing the inhibitory receptor signal. PD-1-CD3 bispecific antibodies have shown efficacy in the treatment of mice with experimental autoimmune encephalomyelitis (EAE) (462) and further studies might reveal whether they are also effective in other inflammatory conditions including RA and PsA. Simultaneous targeting of different inhibitory receptors expressed by the same cell or by different cells might also be a feasible approach. The rationale behind this type of treatment lays in the fact that combined PD-1 and CTLA-4 blockade in cancer patients has additive effect on the incidence and the severity of autoimmune-like adverse effects (199). These different strategies using high-affinity antibodies or artificial ligands have the potential to provide stronger modulation as compared to naturally expressed ligands. They might also provide therapeutic effects in conditions where the natural ligand is not properly functioning, minimally expressed or when its activity is negatively modulated by a soluble form of their specific receptor as shown in this thesis for soluble PD-1. Thus, further research is needed to exploit the full potential of inhibitory receptors in autoimmune conditions.

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8 Appendix

8.1 Publications derived from this work

Bommarito D, Hall C, Taams LS, Corrigall VM (2017). **Inflammatory cytokines compromise programmed cell death-1 (PD-1)-mediated T cell suppression in inflammatory arthritis through up-regulation of soluble PD-1**. *Clin Exp Immunol.* 2017 Jun;188(3):455-466. doi: 10.1111/cei.12949. Epub 2017 Apr 3.

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Inflammatory cytokines compromise programmed cell death-1 (PD-1)-mediated T cell suppression in inflammatory arthritis through up-regulation of soluble PD-1

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Summary

The programmed cell death 1 (PD-1) receptor plays a major role in regulating T cell activation. Our aim was to determine how inflammation influences PD-1-mediated T cell suppression. Flow cytometry analysis of rheumatoid arthritis (RA) and psoriatic arthritis (PsA) synovial fluid (SF) mononuclear cells showed an increase in the percentage of PD-1$^+$ cells within the CD4$^+$ and CD8$^+$ T cell compartment compared to paired peripheral blood (PB). Upon *in-vitro* T cell receptor (TCR) stimulation of healthy control (HC) CD4$^+$ T cells in the presence of plate-bound PD-L1fc chimera, significantly decreased proliferation and interferon (IFN)-γ secretion was observed. In contrast, CD4$^+$ T cells from RA and PsA PB and SF appeared resistant to such PD-1-mediated inhibition. Addition of the proinflammatory cytokines tumour necrosis factor (TNF)α, interleukin (IL)-6 and IL-1β, which were increased in RA and PsA SF compared to osteoarthritis (OA) SF, consistently abrogated PD-1-mediated suppression in HC CD4$^+$ T cell cultures. This effect was reversed by inhibitors of these cytokines. Soluble PD-1 (sPD-1) levels were increased in cell culture supernatants from TNFα and IL-6-stimulated cultures compared to untreated controls, and also in RA and PsA, but not in OA, serum and SF. Functionally, addition of sPD-1fc counteracted PD-1-mediated suppression of HC CD4$^+$ T cells, and increased T cell proliferation in HC CD4$^+$ T cell/monocyte co-cultures. These *in-vitro* findings indicate that CD4$^+$ T cells from patients with RA and PsA show increased resistance to PD-1-mediated suppression, which may be explained in part by the presence of soluble PD-1 in the inflammatory environment.

Keywords: inflammatory arthritis, IL-6, PD-1, PD-L1, TNFα

Introduction

The programmed cell death 1 (PD-1) receptor, a transmembrane protein and member of the B7 family, plays a critical role in T cell regulation [1]. PD-1 is expressed on T cells, where its expression increases within the first 24 h of T cell activation and decreases with antigen clearance [2–4]. Upon ligation of PD-1 by its ligands (PD-L1/B7-H1 and PD-L2/B7-DC), T cell responses are down-regulated [5,6]. PD-1 ligation leads to inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in reduced Akt (protein kinase B) phosphorylation and reduced expression of transcription factors GATA-3, T-bet and Eomes [7,8]. The overall effect of PD-1 ligation is decreased T cell activation and cytokine production [9–12]. The clinical relevance of PD-1 in immune regulation is evidenced by the recent success of PD-1 blockade in the treatment of certain end-stage cancers, leading to reduced tumour burden and enhanced anti-tumour immunity in a considerable number of patients [13,14]. Conversely, in inflammatory conditions it has been documented that disruption of the PD-1 gene (*pdcd1*) in mice leads to lupus-like syndrome, proliferative arthritis, diabetes, autoimmune cardiomyopathy and increased susceptibility to collagen-induced arthritis (CIA) [15–20]. In humans, polymorphisms in the *PDCD1* gene have been associated with susceptibility to rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type 1 diabetes mellitus [21–25].

Several investigators have shown that frequencies of PD-1^+ $CD4^+$ T cells are increased in RA synovial fluid compared to RA peripheral blood (PB) and healthy control (HC) PB [20,26,27]. However, despite high levels of this inhibitory receptor at the site of inflammation, the immune system seems unable to regulate persistent T cell activation and cytokine production. This poses the question as to whether the PD-1 pathway is impaired during inflammation. An indication of a defective PD-1 pathway in RA comes from a study indicating that RA synovial fluid (SF) $CD4^+$ T cells show reduced PD-1-mediated inhibition compared to RA PB cells [20]. This suggests that under conditions of chronic inflammation the PD-1 pathway is modulated. Thus far, little is known regarding the PD-1/PD-L1 pathway in the context of psoriatic arthritis (PsA). PsA and RA, while sharing a number of common pathological features, are two distinct diseases with serological, genetic and radiological differences [28]. Here we determined the expression of PD-1 on T cells from PB and SF of patients with RA or PsA, and investigated how inflammatory mediators associated with RA and PsA affect PD-1-mediated T cell suppression. Our data indicate that $CD4^+$ T cells from patients with RA and PsA are compromised in their PD-1-mediated inhibition and reveal a potential role for soluble PD-1 (sPD-1) in the aberrant PD-1-mediated regulation in these diseases.

Materials and methods

Patients and healthy volunteers

Heparinized PB and matched SF samples were obtained from patients with RA and PsA recruited from the rheumatology out-patient clinic at Guy's and St Thomas' Hospital NHS Trust (London, UK). Information on clinical and demographic parameters is provided in Supporting information, Table 1. HC subjects were recruited from among local student and staff volunteers. Written informed consent was received from all participants. Ethics approval was given by the Bromley Research Ethics Committee (approval no. 06/Q0705/20) for HC, RA and PsA and by the Guy's Research Ethics Committee (approval no. 01/05/01) for osteoarthritis (OA). All samples were collected in compliance with the Declaration of Helsinki.

PBMC, SFMC and cell subset isolation

PB mononuclear cells (PBMC) and SF mononuclear cells (SFMC) were isolated by Lymphoprep^{TM} (Axis-Schield, Oslo, Norway) density-gradient centrifugation. Cell subsets were isolated by magnetic separation (Miltenyi Biotech, Bergisch Gladbach, Germany and Dynabeads Thermofisher, Paisley, UK) and purity was determined by flow cytometry. $CD4^+$ T cells (purity range 95–99%) were isolated by negative isolation from PBMC or SFMC or from the CD14-depleted cell fractions following the manufacturers' instructions. $CD14^+$ monocytes (purity range 96–98%) were selected positively using CD14 MicroBeads (Miltenyi Biotec).

Cell culture

Cell subsets were cultured for 5 days in culture medium (RPMI-1640; Gibco, Paisley, UK), supplemented with 1% penicillin/streptomycin, 1% L-glutamine (Gibco) and 10% heat-inactivated fetal calf serum (Gibco) and maintained at 37°C and 5% CO_2 atmosphere. Cells were stimulated with either plate-bound anti-CD3 monoclonal antibody (mAb) (OKT3; Janssen-Cilag Ltd, High Wycombe, UK) (1·5 μg/ml) in $CD4^+$ T cell only cultures or with soluble anti-CD3 mAb (OKT3; Janssen-Cilag Ltd) (100 ng/ml) in $CD4^+$ T cell/$CD14^+$ monocyte co-cultures.

Flow cytometry analysis of cell frequency and phenotype

For *ex-vivo* analysis of frequency and phenotype of each cell subset, PBMC or SFMC were stained extracellularly for 30 min at 4°C using different combinations of the following antibodies: fluorescein isothiocyanate (FITC)-conjugated CD279 (PD-1; BioLegend, Cambridge, UK), phycoerythrin (PE)-conjugated CD274 (PD-L1; BD Pharmingen, Oxford, UK), PE/cyanin 7 (Cy7)-conjugated CD3 (Biolegend), peridinin chlorophyll (PerCP)/Cy5·5-conjugated CD4 (Biolegend), PacBlue-conjugated CD8 (Biolegend), allophycocyanin (APC)-conjugated CD8 (Biolegend) and Vio770-conjugated CD14 (Miltenyi Biotech). Following surface staining, cells were fixed in 2% paraformaldehyde (PFA) for 15 min at 4°C, washed twice and acquired on a BD fluorescence activated cell sorter (FACS)Calibur or a BD FACSCanto II. Data were analysed using FlowJo software (version 7.6.5; Tree Star, Ashland, OR, USA).

T cell proliferation and PD-1 ligation assays

96 well flat-bottomed plates (Costar, Corning Inc., Corning, NY, USA) were coated with anti-CD3 mAb (OKT3; Janssen-Cilag Ltd) (1·5 μg/ml) and with either PD-L1fc or immunoglobulin (Ig)G1fc (R&D Systems, Abingdon, UK) (ranging from 0 to 5 μg/ml according to the experiment) in phosphate-buffered saline (PBS) solution (Sigma Aldrich, Poole, UK) for 4 h at 37°C and 5% CO_2. Plates were washed twice with PBS before cells were added for culture. $CD4^+$ T cells were isolated from cryopreserved HC PBMC, RA and PsA PBMC and RA and PsA SFMC and plated at a concentration of 1×10^5 cells per well in a final volume of 200 μl of culture medium. In some cultures, human recombinant tumour necrosis factor (hrTNF)α, human recombinant interleukin (hrIL)-6 or hrIL-1β (all at 10 ng/ml; R&D Systems) were added in the absence or presence of anti-TNFα drug adalimumab (AbbVie, Chicago, IL, USA), anti-IL-6R drug tocilizumab (Roche, Basel,

Switzerland) or anti-IL-1β mAb (R&D Systems) (all at 1 μg/ml). In some cultures, HC CD4$^+$ T cells were cultured in PD-L1fc (0, 0·1 and 1 μg/ml)-coated plates in the presence of soluble PD-1fc (0·5 and 1 μg/ml; R&D Systems). In other experiments, freshly isolated HC CD4$^+$ T cells and autologous CD14$^+$ monocytes (used as a source of PD-L1 ligand) were co-cultured in 96-well flat-bottomed plates (Costar, Corning Inc.) at 1 : 1 ratios (total cells per well 1×10^5) in culture medium containing 100 ng/ml soluble anti-CD3 mAb and soluble PD-1fc or IgG1fc (0, 0·25, 0·5 and 1 μg/ml). In all assays, at day 4, cells were pulsed with [^{3}H]-thymidine (0·25 μCi/well) (GE Healthcare, Little Chalfont, UK) and T cell proliferation was assessed after 18 h (on day 5) using a Topcount scintillation counter (Perkin Elmer, Cambridge, UK). Proliferation was determined and expressed as counts per minute (cpm) and as suppression of T cell proliferation (%) according to the following formula: [(medium only condition − PD-L1fc condition)/medium only condition] × 100.

Detection of soluble cytokines and soluble PD-1

CD4$^+$ T cell culture supernatants were collected at day 5 and stored at −80°C. IFN-γ levels were determined by enzyme-linked immunosorbent assay (ELISA) using the ELISA MAX^{TM} standard set (Biolegend). Levels of sPD-1 were determined by human PD-1 DuoSet ELISA (R&D Systems). Serum samples from HC donors and serum and paired cell-free SF samples from patients with OA, RA or PsA were collected and stored at −80°C until analysed by ELISA (for sPD-1; R&D Systems) or Bio-Plex Pro^{TM} (for TNFα, IL-6 and IL-1β; Bio-Rad Laboratories) on the Luminex FlexMap 3D platform (Luminex Corporation, Austin, TX, USA). All assays were performed according to the manufacturers' protocols.

RNA extraction and real-time–quantitative polymerase chain reaction (RT–qPCR)

Total RNA was isolated using the ReliaPrep^{TM} RNA cell Miniprep System (Promega, Southampton, UK) according to the manufacturer's protocol. cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and mRNA expression of PD-1Δex3 was determined using the SensiMix^{TM} SYBR No-ROX Kit (Bioline, London, UK). Data were collected and analysed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany). The β-actin gene was used as an endogenous control and relative gene expression was expressed as $2^{-\Delta\Delta CT}$. PCR primer pairs (IDT, Leuven, Belgium) were as follows: PD-1Δex3, 5′-AGGGTGACAGG GACAATAGG-3′ and 5′-CCATAGTCCACAGAGAACAC-3′, β-actin, 5′-ATTGGCAATGAGCGGTTC-3′ and 5′-CGTGGATGCCACAGGACT −3'.

Statistical analysis

Significance testing was performed with GraphPad Prism software (version 7; GraphPad, La Jolla, CA, USA) using the appropriate statistical tests, as indicated in the figure legends.

Results

PD-1$^+$ T cell frequencies are increased in RA and PsA synovial fluid compared to peripheral blood

First, we investigated the frequencies of PD-1$^+$ cells among T cells in PB and paired SF from patients with RA and PsA. Significantly increased percentages of PD-1$^+$ cells were found within SF CD4$^+$ T cells (identified either by CD3$^+$CD14$^-$CD4$^+$ cells or CD3$^+$CD8$^-$ cells) compared to PB (Fig. 1a,b). In addition, increased percentages of PD-1$^+$ cells were found within the SF CD8$^+$ T cell compartment in both RA and PsA (Fig. 1c,d).

PD-1 ligation reduces proliferation of CD4$^+$ T cells from healthy donors, but not CD4$^+$ T cells from patients with RA or PsA

To investigate whether the PD-1 expression is functional in RA and PsA, we set up a PD-1 ligation assay using anti-CD3 and PD-L1fc (or IgG1fc as control)-coated plates, based on previously described protocols [9,29,30]. As expected, PD-1 ligation resulted in a significant and dose-dependent reduction of healthy control PB-derived CD4$^+$ T cell proliferation, while no effect was observed in the presence of IgG1fc control (Fig. 2a,b). IFN-γ production was also inhibited in a PD-L1fc dose-dependent fashion (Supporting information, Fig. S1a). We then compared PD-1 ligation of PB-derived CD4$^+$ T cells from healthy donors and patients with RA in parallel experiments. In contrast to the suppressive effects on T cell proliferation observed upon PD-1 ligation of CD4$^+$ T cells from healthy donors, CD4$^+$ T cells from patients with RA appeared to be resistant to PD-1-mediated suppression of T cell proliferation (Fig. 2c,d). Next, we cultured CD4$^+$ T cells from the blood and synovial fluid from patients with RA and PsA with increasing doses of plate-bound PD-L1fc. Even at the highest dose of PD-L1fc (5 μg/ml) we did not detect a decrease in T cell proliferation upon PD-1 ligation (Fig. 2e,f). Similarly, when RA and PsA cell culture supernatants were tested for IFN-γ production, we could not detect a consistent decrease in the levels of IFN-γ (Supporting information, Fig. S1b). These data indicate that CD4$^+$ T cells from the blood and synovial fluid of patients with RA or PsA are resistant to PD-1 ligation compared to healthy control cells.

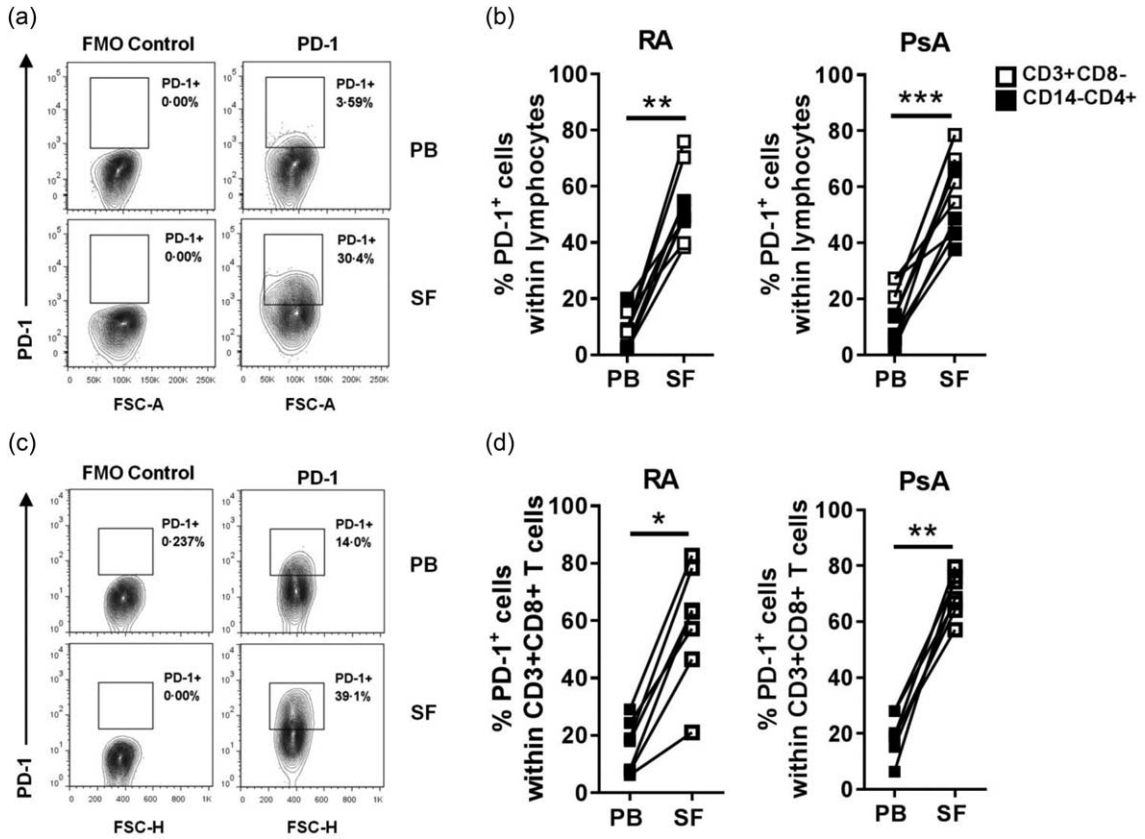

Fig. 1. Programmed cell death 1 (PD-1)$^+$ T cell frequencies are increased in rheumatoid arthritis (RA) and psoriatic arthritis (PsA) synovial fluid compared to peripheral blood. Frequencies of PD-1$^+$ T cells were analysed *ex vivo* by flow cytometry in peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) from RA and PsA patients. (a) Contour plot of CD3$^+$CD14$^-$CD4$^+$PD-1$^+$ cells from paired PBMC and SFMC of one representative PsA donor. (b) Cumulative data showing percentage of PD-1$^+$ cells within CD3$^+$CD8$^-$ or CD3$^+$CD14$^-$CD4$^+$ (RA $n = 10$; PsA $n = 11$) PB and SF cell populations. (c) Contour plot of CD3$^+$CD8$^+$PD-1$^+$ cells from paired PBMC and SFMC of one representative RA donor. (d) Cumulative data showing percentage of PD-1$^+$ cells within CD3$^+$CD8$^+$ (RA $n = 7$; PsA $n = 8$) PB and SF cell populations. Data were analysed by Wilcoxon matched-pairs signed-rank test. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$. Isotype control staining showed a similar result to fluorescence minus 1 (FMO) staining (Supporting information, Fig. S5).

TNFα, IL-6 and IL-1β counteract PD-1-mediated suppression of CD4$^+$ T cell proliferation

Because RA and PsA CD4$^+$ T cells, especially those from the synovial fluid, are derived from a proinflammatory environment, we sought to examine how inflammatory cytokines may influence PD-1-mediated T cell suppression. First, we determined the levels of TNFα, IL-6 and IL-1β in RA and PsA-derived sera and paired SF, compared to healthy serum and serum and SF from disease control patients with osteoarthritis (OA). Increased levels of all three cytokines were detected in both RA and PsA SF when compared to HC serum or OA SF (Fig. 3a,b). These data confirm the inflammatory nature of SF in both RA and PsA. In the analysed RA and PsA sera, we only found mild increases of TNFα and IL-6 compared to healthy or OA control serum.

Next, we assessed whether the presence of TNFα, IL-6 or IL-1β had a functional impact on PD-1-mediated suppression of CD4$^+$ T cell proliferation. HC CD4$^+$ T cells were cultured with increasing concentrations of PD-L1fc (0, 0·1 and 1 μg/ml) in the absence or presence of TNFα, IL-6 or IL-1β (10 ng/ml). To block the effect of the cytokines, the anti-TNFα drug adalimumab, anti-IL-6R drug tocilizumab or anti-IL-1β mAb (1 μg/ml) were added at the beginning of the culture where indicated. Addition of each individual cytokine was able to abrogate the suppressive effects of PD-1 ligation on CD4$^+$ T cell proliferation at both 0·1 and 1 μg/ml PD-L1 concentrations (Fig. 3c). In each sample tested, adalimumab, tocilizumab and anti-IL-1β mAb were able to reverse these cytokine-mediated effects completely. Together, these data indicate that the RA- and PsA-associated inflammatory cytokines TNFα, IL-6 and IL-1β can counteract the suppressive effects of PD-1 ligation on CD4$^+$ T cells, at least *in vitro*.

Soluble PD-1 (sPD-1) is induced *in vitro* by TNFα and IL-6 in HC CD4$^+$ T cell cultures and can be detected in serum and SF of both RA and PsA patients

Having demonstrated that TNFα, IL-6 and IL-1β can be detected in RA and PsA patients and that each of these

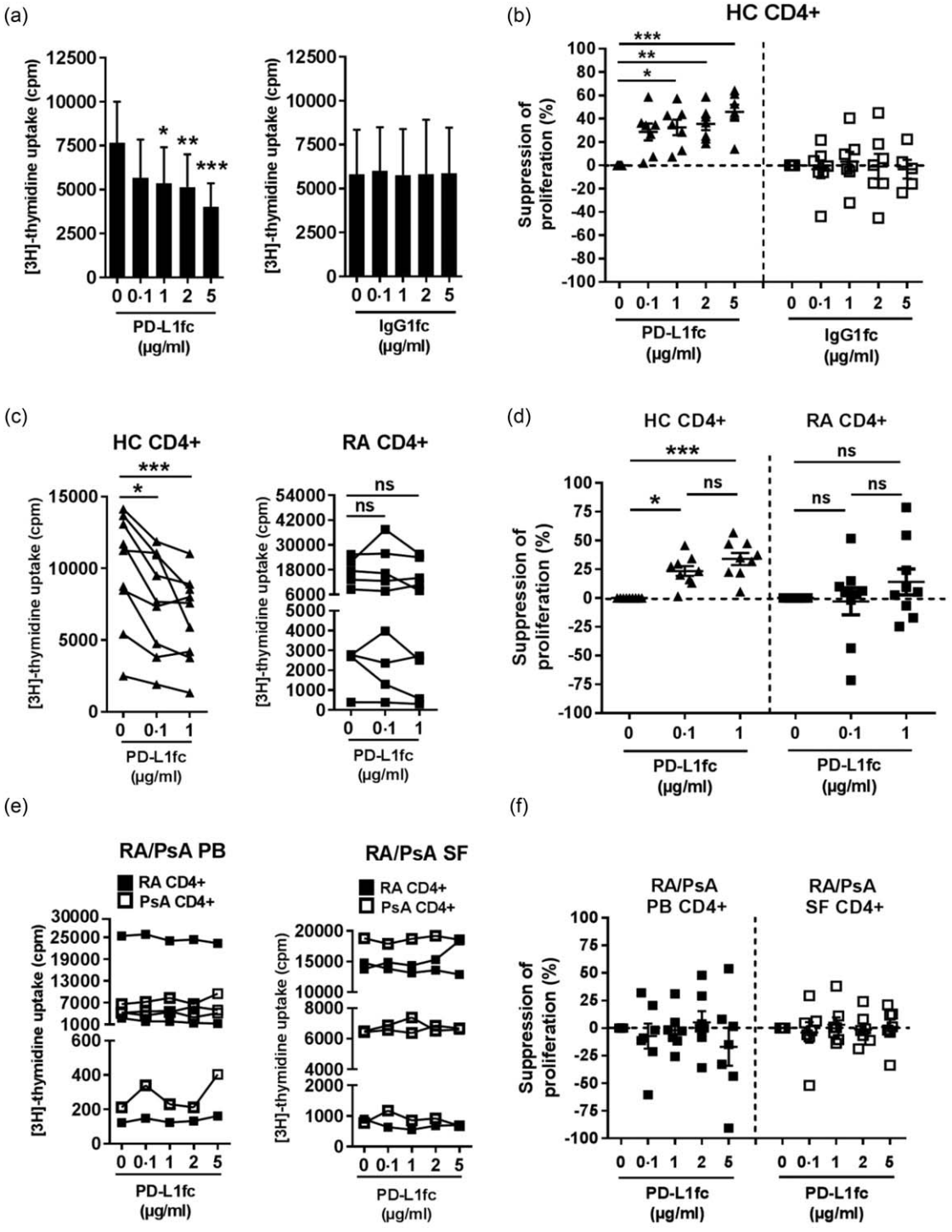

Fig. 2. Programmed cell death-1 (PD-1) ligation reduces proliferation of $CD4^+$ T cells from healthy donors but not $CD4^+$ T cells from patients with rheumatoid arthritis (RA) or psoriatic arthritis (PsA). (a–f) $CD4^+$ T cells were isolated from healthy control (HC) peripheral blood mononuclear cells (PBMC) and RA and PsA PBMC and synovial fluid mononuclear cells (SFMC) and cultured for 5 days in plates precoated with anti-CD3 monoclonal antibody (mAb) (OKT3; 1·5 μg/ml) and PD-L1fc/IgG1fc (0, 0·1, 1, 2 and 5 μg/ml). Proliferation was assessed on day 5 by [^{3}H]-thymidine incorporation. (a) HC $CD4^+$ T cell proliferation (cpm) and (b) suppression of proliferation following PD-1 ligation by PD-L1fc ($n = 7$) or immunoglobulin (Ig)G1fc ($n = 5$–7). (c) Cell proliferation (cpm) and (d) suppression of proliferation of $CD4^+$ T cells isolated from HC and RA PBMC ($n = 9$) in presence of PD-L1fc. (e) Cell proliferation (cpm) and (f) suppression of proliferation of $CD4^+$ T cells isolated from RA and PsA PBMC and paired SFMC in presence of PD-L1fc ($n = 3$ RA PB/SF; $n = 4$ PsA PB/SF). Data were analysed by Friedman test with Dunn's multiple comparison test. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$. Data in (b,d,f) show mean ± standard error of the mean.

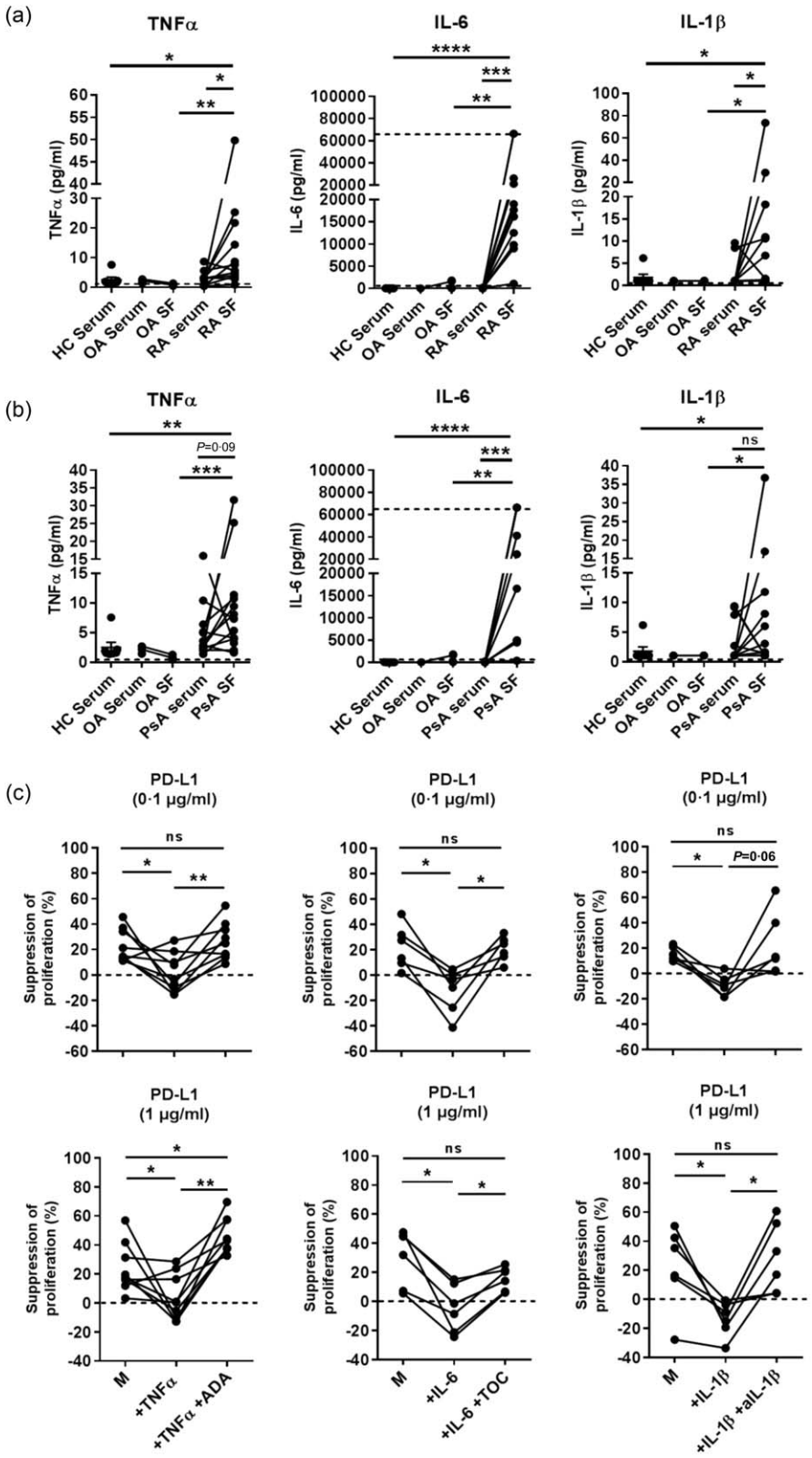

Fig. 3. Tumour necrosis factor (TNF)α, interleukin (IL)-6 and IL-1β counteract the programmed cell death-ligand 1 (PD-L1)-mediated suppression of healthy control (HC) $CD4^+$ T cell proliferation. (a,b) Levels of proinflammatory cytokines TNFα, IL-6 and IL-1β in paired rheumatoid arthritis (RA) and psoriatic arthritis (PsA) serum/synovial fluid (SF) ($n = 12$), in osteoarthritis (OA) (disease control) serum/SF ($n = 3$–4) and in HC serum ($n = 7$). Wilcoxon's matched-pairs signed-rank test for RA/PsA serum *versus* RA/PsA SF and Mann–Whitney test for RA/PsA SF *versus* HC serum or OA SF. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$. (c) Plates were coated with PD-L1fc at the indicated concentrations, and PD-L1-mediated suppression of proliferation by $CD4^+$ T cells from HC PB was assessed in absence (medium, M) or presence of 10 ng/ml of TNFα ($n = 9$), IL-6 ($n = 5$) or IL-1β ($n = 6$) ± anti-TNFα (adalimumab; ADA), anti-IL-6R (tocilizumab; TOC) and anti-IL-1β (all at 1 μg/ml). Data in (c) were analysed by Wilcoxon matched-pairs signed-rank test. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

cytokines abrogates PD-L1fc activity *in vitro*, we sought to identify a possible underlying mechanism. ELISA analysis of HC $CD4^+$ T cell supernatants from cytokine-stimulated cultures revealed that both TNFα and IL-6 were able to induce sPD-1 compared to medium-only conditions (Fig. 4a,b). No significant increase in sPD-1 was observed in IL-1β-stimulated cultures (Fig. 4c). This increase in sPD-1 was abrogated in the presence of adalimumab or

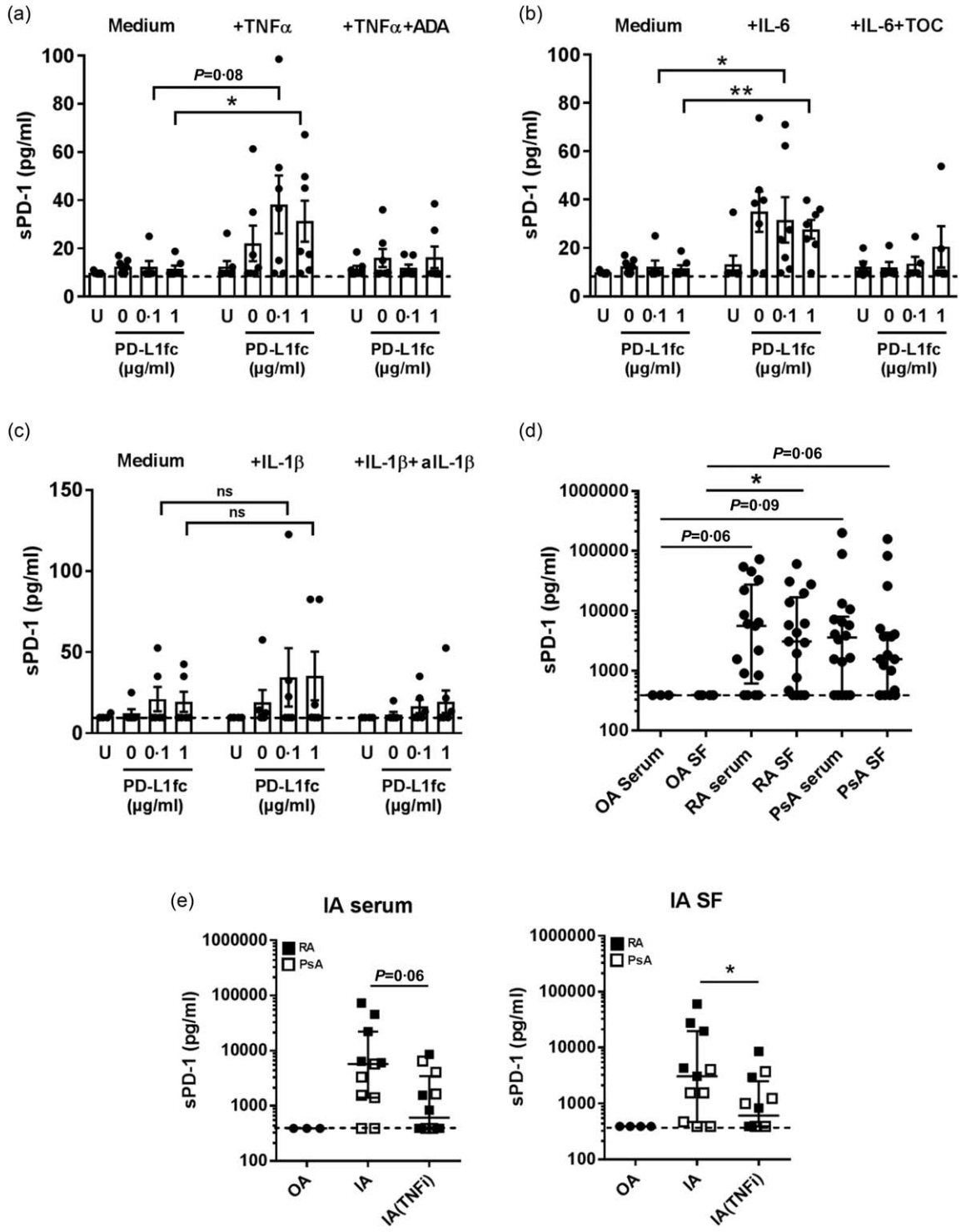

Fig. 4. Soluble programmed cell death-1 (sPD-1) is induced *in vitro* by tumour necrosis factor (TNF)α, interleukin (IL)-6 in HC CD4$^+$ T cell cultures and can be detected in serum and synovial fluid (SF) of rheumatoid arthritis (RA) and psoriatic arthritis (PsA) patients. (a–c) sPD-1 levels in supernatants of healthy control (HC) CD4$^+$ T cells stimulated with (a) TNFα (10 ng/ml) or TNFα+ adalimumab (ADA; 1 μg/ml) (n = 7), (b) IL-6 (10 ng/ml) or IL-6+ tocilizumab (TOC; 1 μg/ml) (n = 5–-7) and (c) IL-1β (10 ng/ml) or IL-1β+ anti-IL-1β (anti-IL-1β; 1 μg/ml) (n = 5). (d) sPD-1 levels (median with interquartile range) in RA and PsA paired serum/synovial fluid (SF) and osteoarthritis (OA) serum/SF (OA, n = 3–4; RA, n = 17; PsA, n = 18). (e) sPD-1 levels (median with interquartile range) in control disease OA (n = 3–4), RA (n = 5) and PsA (n = 6–7) serum and SF of patients treated with TNFi *versus* non-TNFi therapy. Data in (a,b,c,e) were analysed by Mann–Whitney test while data in (d) were analysed by Kruskal–Wallis test with Dunn's multiple comparison test. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

tocilizumab. Quantitative PCR analysis of HC $CD4^+$ T cells from the same TNFα- and IL-6-stimulated cultures revealed increases of the PD-1Δex3 splice variant (Supporting information, Fig. S2). These results are in line with a previous study from another group reporting that TNFα promotes sPD-1 expression [31] but also identify IL-6 as an inducer of sPD-1 and PD-1Δex3.

In addition, ELISA analysis revealed that sPD-1 was detectable in none of three OA serum and none of four OA SF samples, while it was detected at high levels in 13 of 17 RA and 13 of 18 PsA serum and SF samples (Fig. 4d). Additionally, cross-sectional analysis of serum and SF from patients with RA or PsA undergoing TNF-inhibitor (TNFi) therapy revealed lower levels of sPD-1 when compared to patients not receiving TNFi therapy (Fig. 4e). These data show that proinflammatory cytokines such as TNFα and IL-6 can modulate the amount of sPD-1 *in vitro* and that TNFi therapy might modulate sPD-1 levels in the serum and SF of patients with inflammatory arthritis.

sPD-1 modulates PD-1-mediated suppression of HC $CD4^+$ T cells and induces proliferation in $CD4^+$ T cell/$CD14^+$ monocyte co-cultures

To investigate whether sPD-1 is able to modulate PD-1/PD-L1 interactions, we first tested if sPD-1fc itself promotes T cell proliferation of anti-CD3 stimulated HC $CD4^+$ T cells cultured in the absence of PD-L1fc. In these experimental conditions, sPD-1fc did not induce any increase in cell proliferation (Supporting information, Fig. S3), suggesting no direct effect on HC $CD4^+$ T cells. We then cultured HC $CD4^+$ T cells in the presence of increasing amounts of PD-L1fc ligand in the absence or presence of sPD-1fc chimera (0·5 or 1 μg/ml). In PD-L1fc precoated plates, addition of sPD-1fc was able to abrogate the activity of the ligand in a dose-dependent fashion, resulting in less efficient suppression of T cell proliferation when compared to medium only (Fig. 5a,b). These data indicate that in a $CD4^+$ T cell-only culture system, sPD-1 is able to modulate negatively an otherwise functional PD-1/PD-L1 interaction.

To investigate further the ability of sPD-1 to modulate the PD-1/PD-L1 interaction, we set up a co-culture system using HC $CD4^+$ T cells and autologous $CD14^+$ cells as a source of natural PD-L1. The ability of HC $CD14^+$ cells to express PD-L1 was tested by flow cytometry by culturing the cells overnight with 10 ng/ml of IFN-γ, a known inducer of PD-L1 [27,32] (Supporting information, Fig. S4). Freshly isolated HC $CD4^+$ T cells and autologous monocytes were cultured at a 1 : 1 ratio with soluble anti-CD3 mAb (100 ng/ml) in the absence or presence of increasing doses of sPD-1 or IgG1fc control (0·25, 0·5 and 1 μg/ml). Addition of sPD-1fc led to a significant dose-dependent increase in T cell proliferation compared to control-treated cells (Fig. 5c,d). These data indicate that soluble PD-1 receptor can modulate PD-1 ligation in both an artificial system (PD-L1fc precoated plates) as well as in a more physiological context (in the presence of $PD\text{-}L1^+$ APC).

Discussion

The present study provides evidence for compromised PD-1 mediated suppression in $CD4^+$ T cells from patients with RA and PsA. Our study identifies the proinflammatory cytokines TNFα, IL-6 and IL-1β as negative modulators of PD-1-mediated T cell suppression *in vitro* and demonstrates that sPD-1 is capable of interfering with effective PD-1 ligation. To our knowledge, this is the first study to examine PD-1 function in RA and PsA simultaneously, and to provide evidence that the inflammatory milieu of these two diseases has a role in modulating PD-1 ligation.

We show that the frequencies of $PD\text{-}1^+$ cells within $CD4^+$ and $CD8^+$ T cell subsets are increased significantly in the synovial fluid from patients with RA or PsA when compared to peripheral blood, thus extending the findings from previous studies focusing only on RA [20,26,27,33]. The increase in the frequencies of $PD\text{-}1^+$ T cells suggests that in RA and PsA PD-1 might have a role in regulating T cell effectors, especially at the site of inflammation. However, despite this increased frequency of $PD\text{-}1^+$ T cells, inflammation persists, suggesting that this pathway is impaired in these diseases.

Thus far, only limited data exist regarding PD-1 function in inflammatory arthritis. One study using a PD-L1fc chimera demonstrated that PD-1 ligation inhibited cell proliferation and IFN-γ production by $CD4^+$ T cells from peripheral blood of RA patients, but that synovial fluid $CD4^+$ T cells required higher concentrations of PD-L1fc to achieve similar levels of inhibition [20]. The authors speculated that the inflammatory milieu found in the RA joint might be accountable for reduced PD-1-mediated suppression, as they showed that addition of cell-free SF to RA PB $CD4^+$ T cells modulated PD-1 ligation negatively [20]. However, no specific mediator of the effect was identified. It has been shown previously that the common gamma chain cytokines IL-2, IL-7 and IL-15, as well as CD28 co-stimulation, can interfere with PD-1 cross-linking via pSTAT-5 activation [9,29]. In our study, we show that in both RA and PsA, $CD4^+$ T cells from the blood and synovial fluid are more resistant to PD-1 ligation in terms of suppression of T cell proliferation and IFN-γ production when compared to healthy cells. We show that the proinflammatory cytokines TNFα, IL-6 and IL-1β are found at increased levels in RA and PsA SF, and can reduce PD-1-mediated suppression of proliferation in $CD4^+$ T cells from healthy donors. Inhibitors of these cytokines can counteract this effect. These findings provide further evidence that the presence of certain proinflammatory

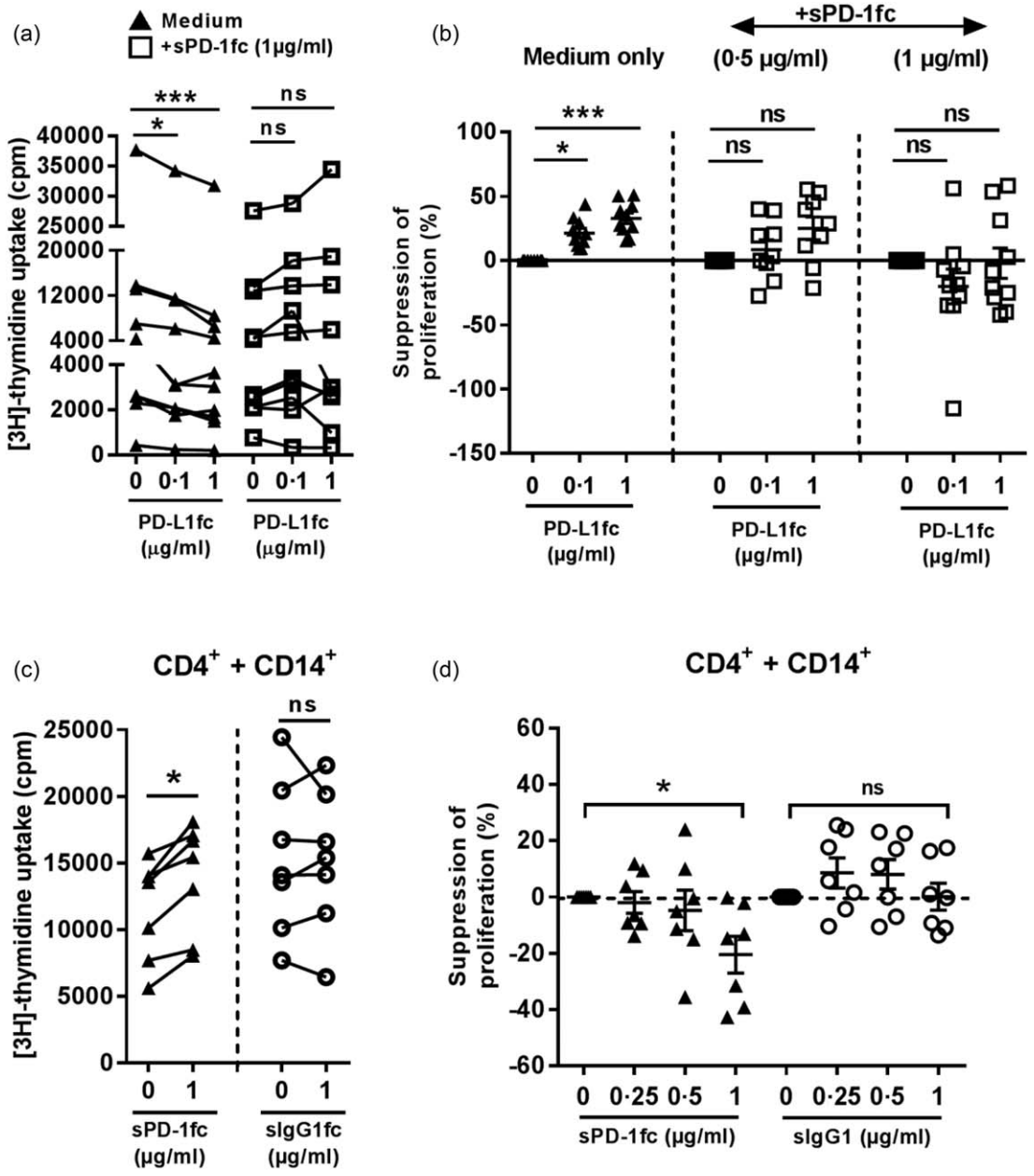

Fig. 5. Soluble programmed cell death 1 (sPD-1) modulates PD-1-mediated suppression of healthy control (HC) $CD4^+$ T cells and induces proliferation in $CD4^+$ T cell/$CD14^+$ monocyte co-cultures. (a) Proliferation and (b) suppression of proliferation of HC $CD4^+$ T cells cultured in anti-CD3 monoclonal antibody (mAb) (OKT3) and PD-L1fc pre-coated plates with or without soluble PD-1fc (0.5 and 1 μg/ml) (n = 9–10). (c) Proliferation and (d) suppression of proliferation of HC $CD4^+$ T cells cultured with autologous $CD14^+$ monocytes at a 1 : 1 ratio (n = 7) in presence of soluble anti-CD3 mAb (100 ng/ml) and soluble PD-1fc/IgG1fc control (0, 0·25, 0·5 and 1 μg/ml). Data were analysed by Friedman's test with Dunn's multiple comparison test (a,b) and Wilcoxon's signed-rank test (c,d). $^*P < 0{\cdot}05$ and $^{***}P < 0{\cdot}001$.

cytokines can be critical in determining the outcome of PD-1 engagement during the immune response.

Mechanistically, our data demonstrate that TNFα and IL-6, but not IL-1β, induce the secretion of sPD-1 by $CD4^+$ T cells, and that sPD-1 levels are increased significantly in the serum and SF of patients with RA or PsA compared to OA. The latter data support and extend two recent studies that showed that sPD-1 can be detected in the serum and SF from patients with RA [27,34]. In these studies sPD-1 serum levels correlated positively with the Disease Activity Score (DAS28), the presence of rheumatoid factor, and with levels of TNFα in the RA SF but not the serum [27,34]. Recent studies showed that expression of the PD-1Δex3 variant is observed in T cells from patients with RA, but only minimally in T cells from patients with OA or from HC [27,31]. PD-1Δex3 is a splice

variant of PD-1, which lacks the transmembrane domain and whose putative translational product is a soluble form of PD-1 [34,35]. It was shown that TNFα, IL-17 and IFN-γ can increase PD-1Δex3 splice variant mRNA expression in healthy human $CD4^+$ T cells [31]. Our data indicate that in addition to those cytokines, sPD-1 protein expression and PD-1Δex3 splice variant mRNA expression can also be regulated by IL-6. Furthermore, preliminary analysis from a cross-sectional investigation of PsA and RA serum and SF suggests that patients treated with TNFi therapy have lower sPD-1 levels compared to patients not treated with TNFi therapy. Further longitudinal studies on patients treated with adalimumab or tocilizumab are required, however, before conclusive statements can be made regarding the effect of biologics on sPD-1 levels.

Our data demonstrate that the inflammatory cytokines TNFα and IL-6 can lead to increased levels of PD-1Δex3 splice variant as well as sPD-1. It is possible that a certain amount of PD-1 might also be released from the cell membrane via other mechanisms. The presence of high levels of certain metalloproteinases (MMPs) such as MMP-9 and MMP-13 has been described previously in inflammatory arthritis [36,37], and it has been shown recently that expression of the PD-1 ligands PD-L1 and PD-L2 in infant foreskin fibroblasts can be regulated through proteolytic cleavage by MMPs [38]. Future studies may reveal whether such an MMP-mediated proteolytic cleavage may also contribute to generation of soluble PD-1.

Notably, we show, using a recombinant PD-1 chimera, that sPD-1 is functionally able to counteract PD-L1fc-mediated suppression of healthy $CD4^+$ T cell proliferation, and to enhance $CD4^+$ T cell proliferation when co-cultured with $CD14^+$ monocytes that can naturally express PD-L1. Importantly, recent studies in autoimmune hepatitis [39] and cutaneous systemic sclerosis [40] support the notion that sPD-1 might interfere with the PD-1 pathway, thereby disrupting T cell regulation.

Collectively, our data indicate that $CD4^+$ T cells from the PB and SF of patients with chronic RA or PsA are more resistant to PD-1-mediated regulation than $CD4^+$ T cells from healthy individuals. We show that the proinflammatory cytokines TNFα, IL-6 and IL-1β, which are present at increased levels in the inflamed joints of RA and PsA patients, are capable of negatively modulating PD-1 ligation *in vitro*. Finally, we show that TNFα and IL-6 are capable of inducing sPD-1 in HC $CD4^+$ T cells and that sPD-1 modulates T cell proliferation by interfering with PD-1 ligation. Thus, our findings provide new evidence that the inflammatory environment of the RA and PsA joint compromises PD-1/PD-L1-mediated T cell regulation.

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Disclosure

The authors have declared no disclosures relating to this study.

Author contributions

D. B. conceived the study, performed most of the experiments and wrote the manuscript. C. H. performed some of the experiments. V. M. C. and L. S. T conceived and supervised the study and wrote the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Supplementary Table 1. Demographic and clinical parameters of the patients included in the study. Some samples were used for flow cytometry or functional assays only, whilst other samples were only used for cytokine detection in serum and SF. Clinical and demographic data are provided, where available. Abbreviations used:

DAS28, disease activity score of 28 joints; DMARDs, disease-modifying anti-rheumatic drugs.

Supplementary Figure 1. PD-1 ligation reduces IFN-γ production by HC CD4+ T cells but not RA or PsA CD4+ T cells. CD4+ T cells from HC PBMC, RA and PsA PBMC and SFMC were cultured in plates pre-coated with anti-CD3 mAb (OKT3) and PD-Llfc/IgGlfc. Supernatants were collected at day 5 and tested by ELISA for IFN-γ production. (a) IFN-γ production in HC CD4+ T cell cultures in presence of PD-L1fc (0, 0·1 and 1 μg/m1 range; n=11 and 0, 0·1, 1, 2 and 5 μg/m1 range; n=4). (b) IFN-γ production in RA and PsA CD4+ T cell cultures; IA PB (RA n=2; PsA n=3) and IA SF (RA n=2; PsA n=4). Data were analysed by Friedman Test with Dunn's Multiple Comparison test. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$.

Supplementary Figure 2. Expression of PD-1Δex3 transcript in activated HC CD4+ T cells in presence of TNFα and IL-6. HC CD4+ T cells were cultured in absence (medium, M) or presence of 10 ng/ml of TNFα (n=4) or IL-6 (n=3) +/- anti-TNFα (adalimumab; ADA) or anti-IL-6R (tocilizumab; TOC) (all at 1 μg/ml) for 5 days. PD-1Δex3 expression was examined by qPCR and normalised to β-Actin housekeeping gene (mean ± SEM).

Supplementary Figure 3. Proliferation of HC CD4+ T cells in presence of increasing sPD-lfc concentrations. HC CD4+ T cells (n=9) were cultured with immobilised anti-CD3 mAb (OKT3) in presence of increasing concentrations of sPD-lfc (0, 0·5 and 1 μg/ml). Proliferation was assessed at day 5 by [^{3}H]-thymidine incorporation and displayed as counts per minute (cpm). Data (mean ± SEM) were analysed by Friedman Test with Dunn's Multiple Comparison test. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$.

Supplementary Figure 4. PD-L1 expression in CD14+ monocytes following IFN-γ stimulation. CD14+ monocytes where positively isolated from HC PBMC and cultured overnight at 37°C in medium only or in medium supplemented with IFN-γ (10 ng/ml). PD-L1 expression was assessed after 12 hrs by flow cytometry. (a) Representative experiment. Shaded histograms represent the isotype control, open histograms indicate the expression profile for PD-L1 with/out IFN-γ stimulation. (b) Cumulative data (n=3).

Supplementary Figure 5. PD 1+ T cell frequencies are increased in synovial fluid compared to peripheral blood. Contour plot of CD3+CD4+PD-1+ cells from paired PBMC and SFMC of one representative RA donor. FMO controls and isotype controls are shown for the CD3+CD4+populations.

